WARWICK MEDICAL SCHOOL

# The Effect of Liraglutide on Microvascular Complications and Cardiovascular Risk in Patients with Type 2 Diabetes

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# DECLARATION

I hereby declare that, this thesis and the experiments described herein to be solely the work of the author, except where stated. No part of this thesis has been, or is being submitted for a degree at any other university.

Subash Chander Sivaraman

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# ABBREVIATIONS

| | |
|---|---|
| ACE-I | angiotensin convertase enzyme inhibitor |
| ACR | albumin/creatinine ratio |
| ANP | atrial natriuretic peptide |
| ARB | angiotensin receptor blocker |
| BMI | body mass index |
| BP | blood pressure |
| DCCT | diabetes control and complications trial |
| DPP4 | di-peptidyl peptidase 4 |
| DRS | diabetic retinopathy study |
| ELISA | enzyme linked immunosorbant assay |
| ETDRS | early treatment diabetic retinopathy study |
| GFR | glomerular filtration rate |
| GIP | glucose dependent insulinotropic polypeptide |
| GLP-1 | glucagon like peptide-1 |
| HbA1c | haemoglobin A1c |
| HOMA | homeostatic model assesment |
| hsCRP | high sensitivity C-reactive protein |

| | |
|---|---|
| IL-1 | interlukin-1 |
| IL-6 | interlukin-6 |
| IL-10 | interlukin-10 |
| LEAD | liraglutide effect and action in diabetes |
| MCP-1 | monocyte chemo attractant protein-1 |
| NYHA | New York heart association |
| sICAM-1 | soluble intercellular adhesion molecule-1 |
| sVCAM-1 | soluble vascular cell adhesion molecule-1 |
| REC | regional ethics committe |
| TGF-β | transforming growth factor-β |
| TNF-α | tumour necrosis factor-α |
| UKPDS | United Kingdom prospective diabetes study |
| VEGF | vascular endothelial growth factor |

**ABSTRACT**

Diabetes is a chronic metabolic disease that leads to vasculopathy involving the small and large blood vessels. Studies on animal models indicate that, GLP-1 analogues might have beneficial effects on the above mentioned complications over and above the improvements due to glycaemic control. The aims of this study were to explore effects of the GLP-1 analogue, liraglutide on the microvascular complications and cardiovascular risk in human subjects with diabetes.

In a retrospective cohort study, I compared a group of patients who received liraglutide with a matched group of subjects with type 2 diabetes on other treatments. The changes in urinary albumin:creatinine ratios and retinopathy grades of these patients were compared after 12 months. In a prospective study, the serum biomarkers of diabetic nephropathy and diabetic retinopathy were measured in a group of subjects with type 2 diabetes, before and 3 months after treatment with liraglutide. In addition body composition analysis of a group of morbidly obese patients with type 2 diabetes was performed before, and 3 months after treatment with liraglutide.

In subjects with established microalbuminuria, treatment with liraglutide for 12 months did not have any effect on albumin excretion rate. Similarly, no changes in urinary albumin:creatinine ratios were evident in normoalbuminuric subjects after 12 months of treatment with liraglutide. I did not find any significant changes in the biomarkers of diabetic nephropathy following treatment with liraglutide.

In patients with type 2 diabetes, therapy with liraglutide for 12 months did not influence the course of diabetic retinopathy. No changes in the serum concentrations of VEGF (which is a biomarker for retinopathy) were noted in patients after treatment with liraglutide.

In morbidly obese subjects with type 2 diabetes, 3 months of treatment with liraglutide induced significant improvements in the markers of cardiovascular risk and reduced total body fat mass. An improvement in the mean insulin sensitivity was noted after treatment; however this did not reach statistical significance.

In conclusion, my study has shown that treatment of patients with type 2 diabetes and obesity with liraglutide was associated with improvements in markers of cardiovascular risk; but no changes in diabetic renal disease or diabetic retinopathy were evident.

# CHAPTER 1

# GENERAL INTRODUCTION

# Diabetes Mellitus

## Prevalence and classification of diabetes

Diabetes mellitus is the most common chronic metabolic disease, and its prevalence continues to increase (Shaw, Sicree et al. 2010, Danaei, Finucane et al. 2011). The increasing global prevalence of obesity is closely associated with the increase in incidence of type 2 diabetes (Vinciguerra, Baratta et al. 2013). It is estimated that there were 285 million people with diabetes worldwide in 2010. This number is projected to increase to increase by 54% by 2030, fuelled by population growth, ageing of populations, urbanisation and associated lifestyle changes (Ramachandran, Snehalatha et al. 1999). There is accumulating evidence that specific lifestyle intervention programs reduce the incidence of type 2 diabetes (Pan, Li et al. 1997, Ramachandran, Snehalatha et al. 2006).

Diabetes mellitus needs on going medical care and self-management by patients. The management is often complex especially in the later stages of the disease and needs to address a number of other medical problems in addition to glycaemic control. Diabetes is classified as (2012)

1. Type1 diabetes due to β cell destruction leading to absolute insulin deficiency
2. Type 2 diabetes due to a combination of insulin resistance and relative insulin deficiency
3. Other specific types of diabetes due to genetic syndromes, drugs and diseases of the exocrine pancreas
4. Gestational diabetes which is first diagnosed during pregnancy

In some patients it might be difficult to distinguish between type 1 and type 2 diabetes at the time of initial presentation. The diagnosis may become more obvious with time.

**Management of diabetes**

Patients with diabetes should be managed by multidisciplinary teams including physicians, diabetes nurses, dieticians, podiatrists and pharmacists. The input from other health professionals may be required in different stages of the disease. In addition, it is vital that patients with diabetes take an active part in self-management (2012). Initial management of diabetes includes appropriate diet, exercise and a healthy life style (National Collaborating Centre for Chronic 2008). Insulin is the mainstay of treatment for glycaemic control in patients with type 1 diabetes and some patients with other specific types of diabetes. A number of other oral and parenteral drugs may be considered for patients with type 2 diabetes.

**Economic burden of diabetes**

The estimated total cost of diabetes in the United States of America in 2002 was $132 billion, which increased to $174 billion in 2007 (Hogan, Dall et al. 2003, Association 2008). By 2012 this figure increased to $245 billion, confirming the continued upward trend in the economic burden of the disease (Association 2013). People with diabetes are more likely to require hospital inpatient care, multiple medications, outpatient visits to physicians and other health professionals. Patients with diabetes are more likely to be admitted to the hospital with other medical problems and are have higher average lengths of stay in hospital (Association 2008). A substantial amount of attributed health resource use is for the chronic complications of diabetes, especially complications related to macrovascular disease and diabetic nephropathy (Association 2013). Estimates from countries in Asia and Europe confirm the increasing economic costs of diabetes and the consequent strain on health resources (Ballesta, Carral et al. 2006, Chatterjee, Riewpaiboon et al. 2011, Marchesini, Forlani et al. 2011).

**Complications of diabetes**

Diabetes may be regarded primarily as a vascular disease that manifests as hyperglycaemia (Aiello, Gardner et al. 1998). The chronic complications of diabetes are broadly classified into microvascular and macrovascular. The clinical manifestations of macrovascular disease include coronary artery disease, cerebrovascular disease and peripheral vascular disease (1998). Indeed a major share of mortality due to diabetes is attributed to macrovascular disease (Association 2008). The clinical presentations of microvascular disease include nephropathy, retinopathy and neuropathy that are associated with significant morbidity due to diabetes (Brinchmann-Hansen, Dahl-Jørgensen et al. 1992).

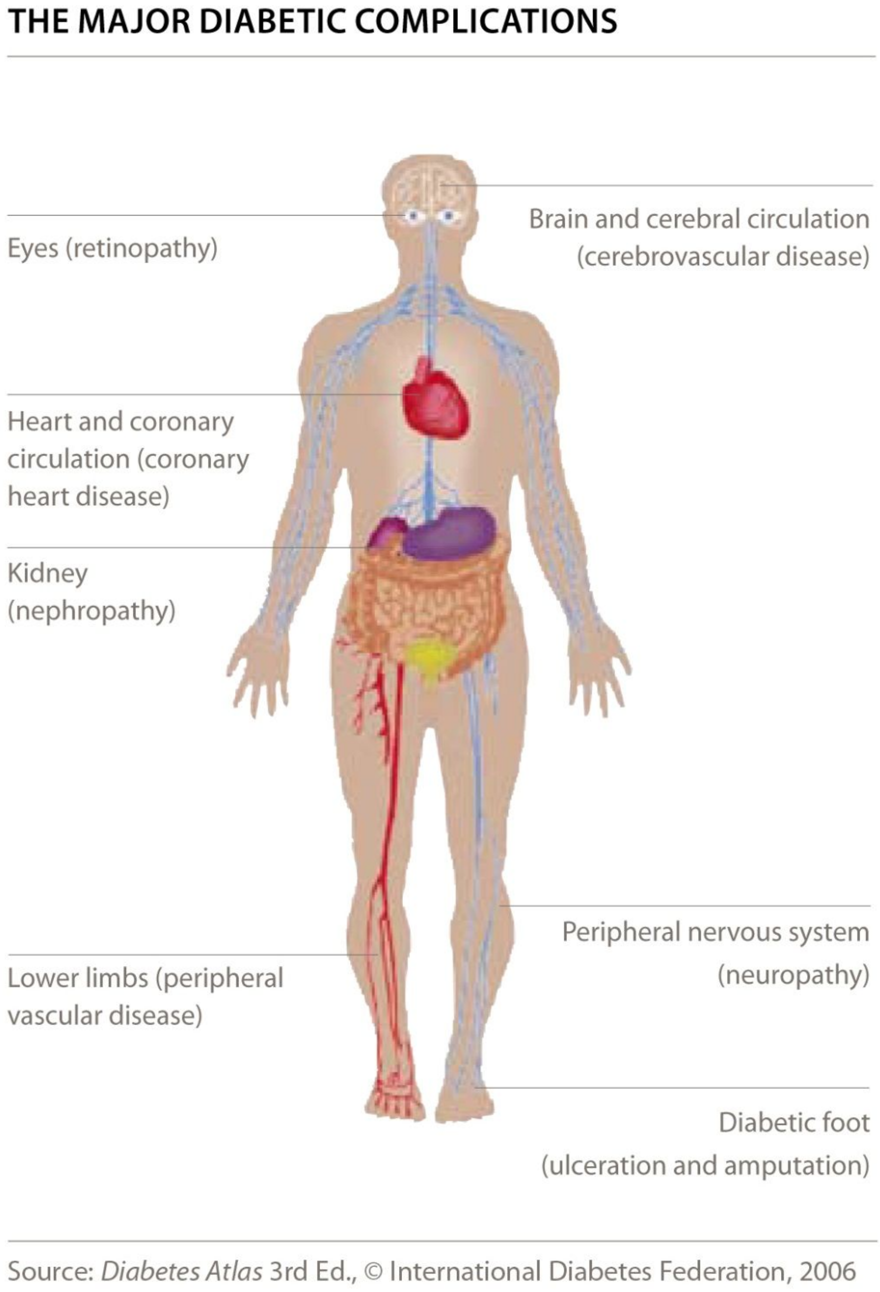



Figure 1.1: CARTOON DEPICTING THE MAJOR CHRONIC COMPLICATIONS OF DIABETES. The microvascular complications nvolve the kidneys, retinae and nerves. The macro vascular complications are manifested as heart attack, stroke or peripheral vascular disease.

## Diabetic nephropathy

### Overview of diabetic nephropathy

Diabetic nephropathy is a major cause of chronic kidney disease, and currently the most common cause of end stage renal failure (Molitch, DeFronzo et al. 2004). Management of patients with chronic kidney disease and diabetes is more complex and expensive than management of patients with diabetes alone (Laliberté, Bookhart et al. 2009). Glomerular hyperperfusion and hyperfiltration herald the onset of nephropathy, followed by leakage of albumin in urine, and subsequently progressive renal failure (Molitch, DeFronzo et al. 2004). Albuminuria is an indicator of renal pathology and also an independent marker of cardiovascular risk (Mann, Yi et al. 2004). There is a further increase in the cardiovascular risk of patients with diabetes that mirrors a decline in the glomerular filtration rate (Ninomiya, Perkovic et al. 2009). About a third of patients with diabetes develop significant nephropathy, and the associated pathologic changes in the kidneys of these patients are complex and heterogeneous (Gambara, Mecca et al. 1993). Poorly controlled diabetes, hypertension and genetic predisposition are associated with progressive renal disease due to diabetic nephropathy (Dronavalli, Duka et al. 2008).

### Screening and diagnosis of diabetic nephropathy

As early stages of diabetes related renal damage are completely asymptomatic, it is recommended that all patients with diabetes are screened annually for urinary albumin excretion in addition to serum creatinine (Rodbard, Blonde et al. 2007). Microalbuminuria is the earliest indicator of renal involvement and has been traditionally defined as 30-300 mg of albumin in a 24 hour collection of urine. Although a 24 hour collection of urine would be ideal in order to account for diurnal variations in albumin excretion, it is often impractical in a clinical setting (2012). The ratio of albumin to creatinine in a spot sample of urine gives comparable results to total albumin excretion in a 24 hour collection (Bakker 1999). Urinary albumin:creatinine ratio in spot urine samples more than 2.5 mg/mmol in men or 3.5 mg/mmol in women are diagnostic of microalbuminuria (National Collaborating Centre for Chronic 2008).

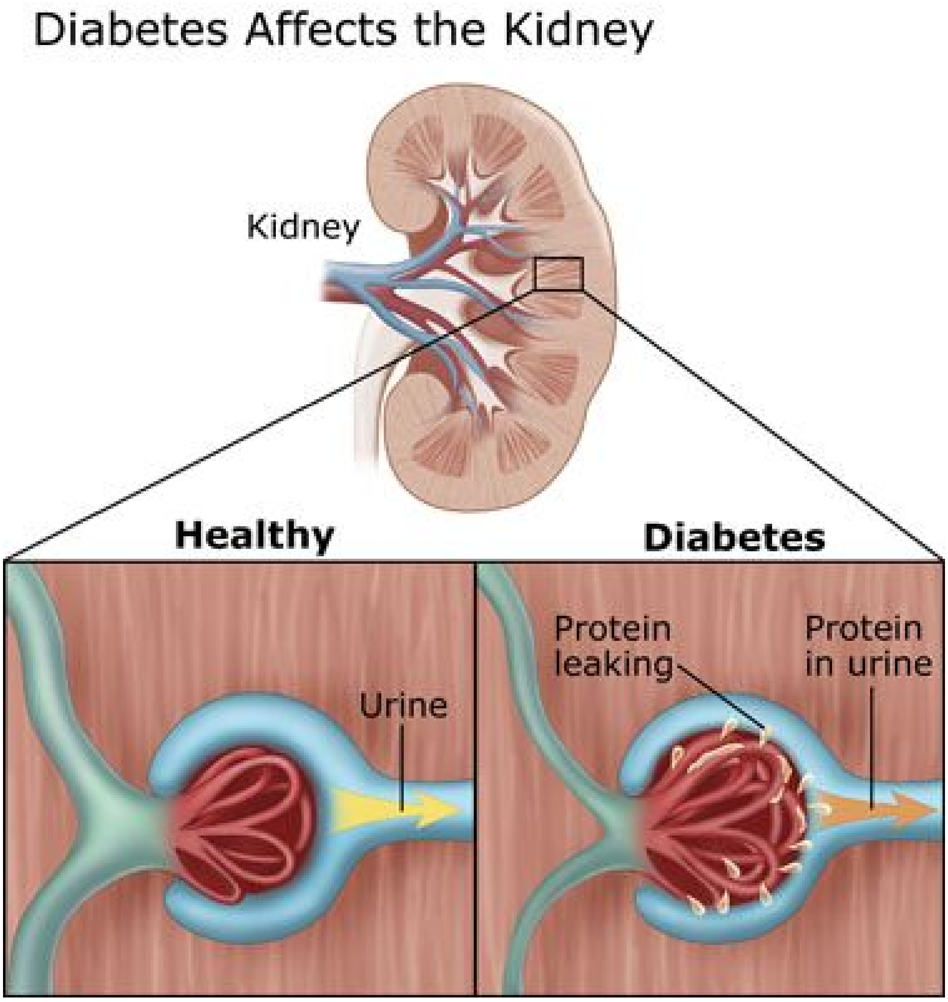



Figure 1.2: CARTOON DEPICTING ALBUMIN LEAKAGE FROM THE GLOMERULUS. It has been postulated that the integrity of the glomerular basement membrane is breached in diabetic nephropathy allowing leakage of large proteins form the plasma into the renal tubules.

## Management of diabetic nephropathy

### Glycaemic control

The UK prospective diabetes study is one of the largest clinical studies conducted on patients with type 2 diabetes. Appropriately matched cases and controls were randomised to either tight glycaemic control (HbA1c of 7%) or usual treatment (HbA1c of 7.9%). After 9 years there was a significant reduction in the relative risk of microalbuminuria ( 0.76, 99% confidence interval 0.62-0.91, p<0.001) and the relative risk of doubling of serum creatinine ( 0.40, 99% confidence interval 0.14-1.20, p=0..27) in the group of patients on the tight glycaemic control arm (1998). Early and intensive glycaemic control was associated with a 24% relative risk reduction in the long term risk of composite microvascular end points including diabetic nephropathy (Holman, Paul et al. 2008).

These observations were confirmed in the diabetes control and complications trial (DCCT), which had a similar design, but involved patients with type 1 diabetes (2000). Intensive glycaemic control was associated with a 39% reduction (95% confidence interval 21%-52%) in microalbuminuria and a 54% reduction in albuminuria (95% confidence interval 19%-74%). Hence it is recommended that patients with diabetes are treated with lifestyle measures and appropriate drugs aiming for HbA1c of 7% or less without inducing hypoglycaemic episodes (2012).

**Management of blood pressure**

Appropriate anti-hypertensive treatment is an important aspect of the overall management of patients with incipient and established diabetic nephropathy (Cooper 1998). It has been shown that stabilization of urinary albumin excretion and attenuation of decline in glomerular filtration rate (GFR) correlate with the mean arterial pressure (Jerums, Allen et al. 2004). In one arm of the UKPDS study, patients were randomized to tight blood pressure control (144/82 mm Hg) or normal treatment (154/87). A clinically important reduction in the urinary albumin excretion was seen in the tight blood pressure control group (1998).

Inhibition of the renin-angiotensin-aldosterone axis might favourably influence the glomerular haemodynamics in patients with diabetes (Lewis, Hunsicker et al. 1993). Angiotensin-2 has been shown to exert a tonic constrictive effect on the efferent arteriole, thereby increasing the intraglomerular pressure and leading on to leak of albumin in the urine. Indeed inhibitors of the angiotensin convertase enzyme and blockers of the angiotensin receptor are effective in slowing the progression of microalbuminuria (Lewis, Hunsicker et al. 1993, Brenner, Cooper et al. 2001, Chan, Wat et al. 2004). A recent systematic review concluded that most of the renoprotective effect of these classes of drugs is due to the reduction in blood pressure (Casas, Chua et al. 2005).

## Recent advances in pathogenesis

### Inflammation in nephropathy

Diabetic nephropathy has been traditionally considered as a non-inflammatory condition, but recent studies have identified inflammation as a key component in the initiation and progression of its pathology. Macrophage activation is a key event in experimental models of diabetes and in human subjects (Chow, Ozols et al. 2004). This is associated with glomerular immune complex deposition, chemokine production, progressive fibrosis, eventually leading to glomerular sclerosis (Furuta, Saito et al. 1993, Galkina and Ley 2006). Conversely, experimental studies impairing leucocyte recruitment in the kidney have shown a reduction in albuminuria, in addition to a reduction in glomerular and tubulointerstitial injury (Chow, Nikolic-Paterson et al. 2005). Accumulation of T-lymphocytes in the kidney has been noted in human subjects with type 1 diabetes and early diabetic nephropathy (Moriya, Manivel et al. 2004). Advanced glycation end products bind to specific receptors on lymphocytes, inducing production of interferons and recruitment of macrophages (Imani, Horii et al. 1993). A number of inflammatory cytokines produced by endothelial cells and leucocytes are implicated in the pathogenesis of nephropathy (Lim and Tesch 2012)

### Endothelium in the kidney

The endothelium lines the entire circulatory system and covers up to 7000 m$^2$ of surface area, making it one of the largest organs in the human body. The physiologic functions of the endothelium differ in different organ systems(Aird 2003). The kidneys receive about 25% of the cardiac output at rest via the main renal arteries, which divide into segmental arteries and later into arcuate arteries, interlobular arteries and finally to afferent arterioles. The glomerular capillaries receive blood from the afferent arterioles. Ultrafiltration of plasma occurs in the glomerulus through the fenestrations in the capillary endothelial cells(Brodsky and Goligorsky 2012). As in other tissues, endothelium in the kidney is located in close proximity to and in constant dialogue with other cell types (Thomas, Witting et al. 2008). Endothelial dysfunction, especially in the glomerulus is an important step in the early pathogenesis of diabetes related renal injury (Nakagawa, Segal et al. 2007, Ziyadeh and Wolf 2008). Blood levels of soluble Intercellular Adhesion Molecule-1 (sICAM-1) and soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1) are well validated biomarkers of endothelial dysfunction (Gearing, Hemingway et al. 1992, Witkowska and Borawska 2004).

## Biomarkers of diabetic nephropathy

### sICAM-1

ICAM-1 consists of five extracellular domains and is expressed on the surface of endothelial cells (Rothlein, Mainolfi et al. 1991, Fonsatti, Lamaj et al. 1999). The sICAM-1 in blood represents a circulating form of the protein and reflects the degree of expression of ICAM-1 on the cell surface(Leeuwenberg, Smeets et al. 1992). The release of sICAM-1 is induced by pro inflammatory cytokines such as Tumour Necrosis Factor-α (TNF-α), Interlukin-1 (IL-1) and Interlukin-6 (IL-6), whereas it is inhibited by Interlukin-10 (IL-10), insulin and antioxidants(Witkowska and Borawska 2004). In vitro studies suggest that ICAM-1 might promote angiogenesis by stimulating endothelial cell migration and endothelial cell tube formation(Gho, Kleinman et al. 1999).

**sICAM-1 in diabetic nephropathy**

In an experimental study, sICAM-1 intact and deficient db/db mice with equivalent hyperglycaemia and obesity were compared for renal end points. The sICAM-1 deficient mice had a 77% reduction in albuminuria at 6 months. In addition these mice had significant reductions in glomerular leucocyte infiltration which was associated with reduced glomerular hypertrophy(Chow, Nikolic-Paterson et al. 2005). When diabetes was induced by streptozocin in sICAM-1 intact and deficient mice, glomerular hypertrophy and mesangial matrix expansion were significantly lower in the latter group of mice. The sICAM-1 deficient mice expressed lower levels of Transforming Growth Factor-β (TGF-β) and type-4 collagen in their glomeruli(Okada, Shikata et al. 2003). Wistar fatty, a genetically obese rat strain is an animal model for type 2 diabetes. sICAM-1 was expressed on the glomeruli of the rats and the expression was associated with development of nephropathy(Matsui, Suzuki et al. 1996). These studies indicate that sICAM-1 is critically involved in the pathogenesis of diabetes related renal disease.

**sVCAM-1**

VCAM-1 is present in plasma as a soluble form, sVCAM-1(Arora, Gunther et al. 2010). The plasma sVCAM-1 is a useful biomarker of endothelial stress because, its levels increase with activation of endothelium (Gearing, Hemingway et al. 1992, Pizzolo, Vinante et al. 1994). VCAM-1 is a member of the immunoglobulin family of proteins and exists in two forms in humans (Cybulsky, Fries et al. 1991). VCAM-1 expression in endothelial cells can be induced mechanically by turbulent blood flow or by the effect of inflammatory cytokines (Carluccio, Ancora et al. 2007). VCAM-1 functions as a scaffolding for leucocyte migration and as a signalling mediator through generation of reactive oxygen species(Cook-Mills, Marchese et al. 2011).

**sVCAM-1 in diabetic nephropathy**

There is evidence from animal and human studies suggest that sVCAM-1 is involved in the pathogenesis of diabetic nephropathy. In an experimental study on spontaneously diabetic KKAy mice, the expression of sVCAM-1 in the kidney was assessed after development of albuminuria. The renal interstitium was infiltrated by monocytes, lymphocytes and plasma cells indicating on going inflammation associated with diabetic renal disease. There was increased expression of sVCAM-1 by the infiltrating cells and endothelial cells of venules, suggesting that sVCAM-1 expression contributes to the interstitial inflammation(Ina, Kitamura et al. 1999). Treatment of diabetic rats with experimental anti-inflammatory agents resulted in an inhibition of diabetes induced hypertrophy of the glomeruli. This was associated with a decrease in the expression of pro-inflammatory factors such as sVCAM-1, sICAM-1 and MCP-1 in the renal tissue, suggesting that these molecules are involved in the inflammatory process(Wang, Li et al. 2008).

In a cross sectional study involving 95 human subjects with type 2 diabetes, circulating sVCAM-1 levels were correlated with markers of microvascular (nephropathy, retinopathy and neuropathy) disease. On multivariate regression analysis, only diabetic nephropathy was associated with serum sVCAM-1 concentration. The sVCAM-1 levels correlated with urinary albumin excretion in patients with normal serum creatinine (Koga, Otsuki et al. 1998). It has been noted that sVCAM-1 levels are higher in albuminuric patients with type 2 diabetes when compared with matched group of subjects with normal urinary albumin excretion (Murakami, Tamasawa et al. 2001, Rubio-Guerra, Vargas-Robles et al. 2007).

**Monocyte Chemo attractant Protein -1 (MCP-1)**

Chemokines constitute a family of chemo attractant cytokines that play a major role in selectively recruiting monocytes, neutrophils, and lymphocytes, as well as in inducing chemotaxis (Deshmane, Kremlev et al. 2009). MCP-1 is one of the key chemokines that regulates migration and infiltration of monocytes/macrophages (Cochran, Reffel et al. 1983). Monocyte migration across the endothelium is required for immunological surveillance and for mounting an inflammatory response. MCP-1 is produced by many cell types, including endothelial cells, fibroblasts, epithelial cells, smooth muscle, mesangial cells, astrocytes, monocytes, and microglial cells. But the major source of MCP-1 are monocyte/macrophages (Cushing, Berliner et al. 1990, Standiford, Kunkel et al. 1991, Brown, Strieter et al. 1992). MCP-1 has been shown to be a potential intervention point for the treatment of various autoimmune diseases, including multiple sclerosis (Sørensen, Ransohoff et al. 2004), rheumatoid arthritis (Hayashida, Nanki et al. 2001) and diabetes(Sartipy and Loskutoff 2003).

**Diabetic nephropathy in MCP-1 knockout mouse models**

Diabetic nephropathy involves a renal inflammatory response mediated by macrophages that accumulate in kidneys in association with the local up regulation of monocyte MCP-1. A study examined the progression of streptozocin induced diabetic nephropathy in mice with deficiency of MCP-1. Renal pathology was examined at 2, 8, 12 and 18 weeks after STZ treatment in MCP-1 intact (+/+) and deficient (-/-) mice. Diabetes of 18 weeks duration resulted in albuminuria and renal failure in MCP-1(+/+) mice, but MCP-1(-/-) mice were largely free of these aspects of renal injury. This suggests that MCP-1-mediated macrophage accumulation and activation plays a critical role in the development of diabetic nephropathy in a mouse model(Chow, Nikolic-Paterson et al. 2006).

Another study explored the role of MCP-1 in the progression of renal injury in obese db/db mice that were deficient in the gene encoding MCP-1. The incidence and development of type 2 diabetes were similar in MCP-1(+/+) and MCP-1(-/-) db/db mice between 8 and 32 weeks of age. However, kidney macrophage accumulation and the progression of albuminuria and renal fibrosis were substantially reduced in MCP-1(-/-) mice when compared with MCP-1(+/+) db/db mice. Hence, MCP-1 plays a critical role in inflammation of the kidney, during the course of type 2 diabetes(Chow, Nikolic-Paterson et al. 2007).

**Role of MCP-1 antagonists in mouse models of diabetic nephropathy**

In a mouse model of diabetic nephropathy, the role of MCP-1 antagonist treatment was assessed by blocking its downstream mediators. In this study, meningeal matrix expansion, type IV collagen, transforming growth factor-beta1 positive area, and macrophage infiltration in glomeruli were measured at baseline and after 12 weeks. It was shown that blocking the MCP-1 pathway ameliorated glomerulosclerosis, indicating that the pathway plays a crucial role in the progression of diabetic nephropathy(Kanamori, Matsubara et al. 2007). In another study, MCP-1 was blocked by mNOX-E36-3'PEG which is an anti-Ccl2 L-enantiomeric RNA aptamer that binds with murine MCP-1 and blocks the recruitment of macrophages to the kidneys of db/db mice with type 2 diabetes. The antagonist was injected subcutaneously three times per week into db/db mice with advanced glomerulopathy from 4 to 6 months of age. It was noted that mNOX-E36-3'PEG reduced the number of glomerular macrophages by 40% compared with control db/db mice. This was associated with protection from diffuse glomerulosclerosis and significantly improved the glomerular filtration rate suggesting a key role for MCP-1 in mediating progression of nephropathy (Ninichuk, Clauss et al. 2008).

**High sensitivity C-reactive protein (hsCRP)**

C-reactive protein is produced in the liver during acute and chronic inflammatory processes (Szalai and McCrory 2002). Its levels can rise up to a thousand fold in 48 hours following a sufficiently large inflammatory insult (Gabay and Kushner 1999). C-reactive protein has a potential anti-microbial role by virtue of its ability to bind to the bacterial cell wall (Abernethy and

Avery 1941). Recently it has been recognised that CRP is a useful marker of low grade inflammation associated with a number of vascular diseases (Libby, Ridker et al. 2002). Indeed, there is some evidence that CRP might activate endothelial cells to induce production of adhesion molecules and chemotactic factors (Pasceri, Willerson et al. 2000, Pasceri, Cheng et al. 2001). It has been shown that within person variability of high sensitivity CRP is low. Hence it is a biologically stable marker of vascular dysfunction, providing that the patient is not having an active infection or taking medications that might affect inflammatory responses (Ridker, Rifai et al. 1999).

## Diabetic retinopathy

### Overview of diabetic retinopathy

Diabetic retinopathy is the most frequent cause of blindness in adults (Aiello, Gardner et al. 1998). Twenty years after diagnosis, nearly all patients with type 1 diabetes and at least 60% of patients with type 2 diabetes will have evidence of retinopathy (Fong, Aiello et al. 2004). Diabetic retinopathy with visual loss has a profound negative impact on the lives of patients (Coyne, Margolis et al. 2004). Studies have shown that diabetic retinopathy with mild or negligible visual impairment is also associated with poor quality of life (Klein, Moss et al. 2001).

The early stages of retinopathy are characterised by increased vascular permeability that progresses to ischemia and neovascularisation of retinal microvasculature (Fong, Aiello et al. 2004). Clinically retinopathy progresses from background changes leading to pre-proliferative stage and eventually to proliferative retinopathy.

**Screening for diabetic retinopathy**

The NHS diabetic eye screening program in the UK systematically screens all patients over the age of 12 in the country with diabetes (Scanlon 2008). About 80 local screening programs deliver screening across England coordinated and led by the screening program centre in Gloucester. In 2008, it was estimated that there were two million people with diabetes over the age of 12 and an annual incidence of blindness of 1280 among this population (Scanlon 2008).

Screening is performed by digital retinal photography, which is graded by appropriately trained graders. Retinopathy is graded into four levels: none (R0), background retinopathy (R1), pre-proliferative retinopathy (R2) and proliferative retinopathy (R3). Maculopathy is graded as absent (M0) or present (M1). Similarly, previous photocoagulation is graded as absent (P0) or present (P1). The presence of sight threatening maculopathy, pre-proliferative retinopathy or worse is considered as sight threatening diabetic retinopathy (Harding, Greenwood et al. 2003). After grading patients are referred for appropriate treatment if required, according to agreed pathways (Leese, Morris et al. 2005).

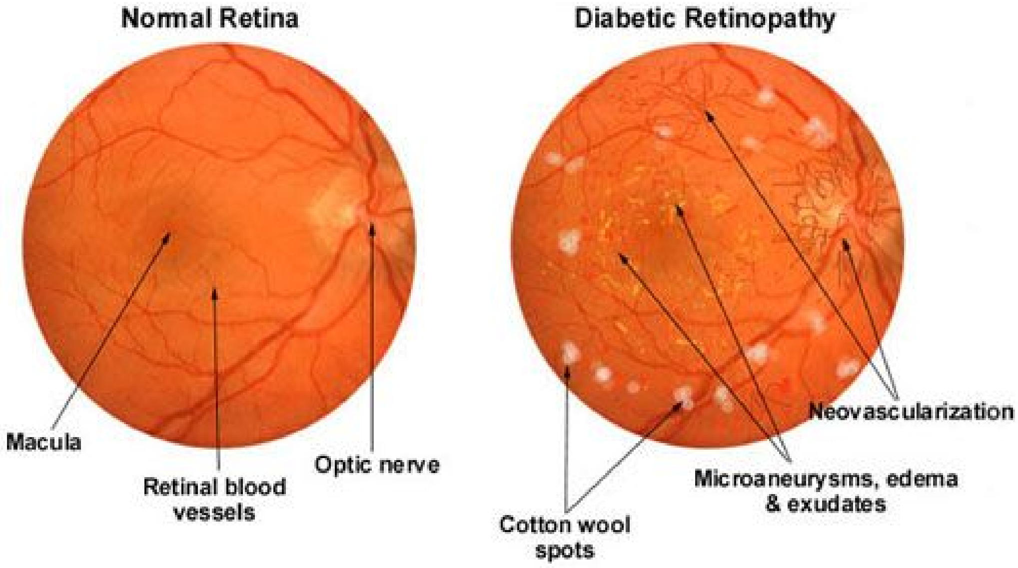



Figure 1.3: RETINAL PHOTOGRAPHS SHOWING NORMAL RETINA AND RETINA WITH DIABETIC RETINOPATHY

**Management of diabetic retinopathy**

**Management of glycaemia and hypertension**

In the DCCT trial 1441 patients with type 1 diabetes, who either had no retinopathy or minimal to moderate non-proliferative retinopathy, were randomised to receive conventional or intensive treatment (1993). Intensive therapy slowed the onset of retinopathy by 76% (95% confidence interval, 62-85%) and progression of retinopathy by 54% (95% confidence interval, 39-66%) (Nathan, Zinman et al. 2009). The protective effect of glycaemic control on retinopathy has also been confirmed for patient with type 2 diabetes. In the UKPDS trial, a continuous relationship was noted between glycaemia and microvascular complications. For every percentage point reduction in HbA1c, there was a 35% relative risk reduction in microvascular complications (Fong, Aiello et al. 2004). Hence tight glycaemic control, with avoidance of hypoglycaemia is an integral part of management of diabetic retinopathy.

Long term follow up data from participants in the UKPDS trial suggests that hypertension is closely linked to the progression of retinopathy (Matthews, Stratton et al. 2004). The relative risk of deterioration in the retinopathy scores of 2 or more (on the ETDRS scale) was lower (0.75, p=0.02) in patients who had tight blood pressure control. In addition, the absolute incidence of blindness in one eye was 3.1/1000 patient years in the tight blood pressure group, versus 4.1/1000 patient years in the conventional blood pressure treatment group (p=0.046, Relative risk 0.76; 99% confidence interval 0.29-1.99). In addition to glycaemic control, treatment of blood pressure is a vital component of management of diabetic retinopathy.

**Laser photocoagulation**

Pan retinal laser photocoagulation is an established treatment for the management of non-proliferative and proliferative diabetic retinopathy (Frank 2004). The diabetic retinopathy study (DRS) randomised patients with bilateral non-proliferative retinopathy or proliferative retinopathy in at least one eye to receive pan-retinal photocoagulation or no treatment (1981). After 2 years there was a significant reduction in the rate of severe visual loss in the treated eyes. The greatest benefits (up to a 50% reduction in relative risk of severe visual loss) were seen in eyes with high risk characteristics. The early treatment in diabetic retinopathy study randomised patients with less severe diabetic retinopathy and visual acuity greater than 20/100 to early laser therapy or deferred treatment (1991). Early photocoagulation reduced the relative risk of severe visual loss by 50% (1991). However the incidence of severe visual loss was low in both groups (2.6% versus 3.7%).

**Newer treatments for retinopathy**

The recently licenced anti-VEGF therapies are a significant addition to our armamentarium for management of diabetic macular oedema. The first anti-VEGF drug, Bevacizumab was initially licenced as a drug to restrict vascular growth in the management of colorectal cancer(Yilmaz, Cordero-Coma et al. 2011). This drug targets all isoforms of VEGF and is currently widely used as an unlicensed treatment for diabetic macular oedema(Goyal, Lavalley et al. 2011). Ranibizumab is a fragment of the same parent molecule as bevacizumab and is licenced for the management of macular degeneration (Nguyen, Shah et al. 2009, Nguyen, Shah et al. 2010). There are no studies directly comparing the efficacies of these two drugs in the management of diabetic macular oedema. However, an indirect comparison by systematic review of clinical trials concluded that there were no significant differences in the efficacies of the two drugs (Ford, Elders et al. 2012).

**Pathologic processes in retinopathy**

**Vascular endothelial growth factor (VEGF)**

VEGF was first identified by Senger et al in 1983 and exists in several isoforms termed as VEGF-A to D (Senger, Galli et al. 1983, Salcedo, Medina et al. 2005). Of these VEGF-A is the most widely studied and implicated in a number of disease processes (Silha, Krsek et al. 2005). VEGF belongs to the family of platelet derived growth factors, the gene for which is located on the short arm of chromosome 6 (Takahashi and Shibuya 1997, Ferrara 2000). VEGF is expressed in a number of tissues including lung, heart, kidney, liver, brain, adrenals and gastric mucosa (Yamaguchi, Yano et al. 1998, Shi, Wang et al. 2001). Its physiological actions include increased vascular permeability and angiogenesis (Yamaguchi, Yano et al. 1998).

**VEGF in diabetic retinopathy**

A number of cell types in the retinal produce VEGF, out of which the Muller cells and astrocytes generally produce the greatest amounts under hypoxic conditions (Aiello, Avery et al. 1994, Hata, Nakagawa et al. 1995). Diabetic retinopathy is characterised by an initial period of vascular injury and increased vascular permeability, which is followed by neovascularisation (Aiello, Gardner et al. 1998). All these changes in the retinal microvasculature are associated with increased expression of VEGF (Duh and Aiello 1999). High glucose levels are linked to oxidative stress leading to increased production of VEGF (Kuroki, Voest et al. 1996, Ellis, Grant et al. 1998). There is a strong correlation between ocular VEGF levels and development of proliferative diabetic retinopathy (Duh and Aiello 1999). Furthermore, the increased vascular permeability due to diabetes can be prevented or reversed by blocking the VEGF pathway (Avery, Pearlman et al. 2006, Haritoglou, Kook et al. 2006).

It is still unclear how the expression of VEGF gene is regulated in the setting of diabetes. Mice studies have shown that hypoxia induces an up regulation of the gene, but this effect is seen as early as a week after hypoxic insult (de Gooyer, Stevenson et al. 2006). This is long before any microscopic changes in the retina are evident. In addition to hypoxia, a number of inflammatory mediators such as TNF-α, TGF-β and growth factors such as IGF-1 also induce up regulation of VEGF (Pertovaara, Kaipainen et al. 1994, Goad, Rubin et al. 1996, Ryuto, Ono et al. 1996). This VEGF expression is mainly the result of paracrine effects of the above mentioned factors.

## Cardiovascular risk in type 2 diabetes

### Markers of cardiovascular risk

It is well recognised that patients with diabetes are at high risk of adverse cardiovascular events. Current management of patients with diabetes involves managing the risk factors for cardiovascular disease in addition to glycaemic control (2012). Hypertension, dyslipidaemia, smoking and obesity are well validated markers of macrovascular disease (Cleary, Orchard et al. 2006). It has been recognised recently that, body composition and insulin resistance are sensitive markers of cardiovascular risk (Tai, Lau et al. 2000).

### Body composition

Recently it has been recognised that adipose tissue is a complex endocrine organ, possibly the largest in the human body, that releases a number of adipokines that influence metabolic and cardiovascular risk factors (Kershaw and Flier 2004). Total body fat mass, percentage of body fat and body fat distribution, especially the degree of hepatic adiposity influence insulin resistance and thereby modulate the risk of macrovascular complications in subjects with diabetes (Kissebah, Vydelingum et al. 1982, Sato, Tamura et al. 2007, Vitola, Deivanayagam et al. 2009, Solomon, Haus et al. 2010). A number of techniques are available to estimate body composition such as bio-electrical impedance, dual energy X-ray absorptiometry, quantitative computer tomography, magnetic resonance spectroscopy and air displacement plethysmography (Lee and Gallagher 2008). The choice of technique depends on local availability and suitability to the experimental study.

**Bio-electrical impedance analysis**

Bio-electrical impedance analysis is a commonly used technique that measures the resistance to a small electrical current as it travels through the total body's water pool (Lee and Gallagher 2008). An estimate of total body water is acquired from which total body fat free mass is calculated. The most commonly used technique is single frequency bio-impedance analysis that provides limited data about body composition. Advances in technology had led to multi-segmental bio-impedance spectroscopy that gives more sophisticated data about fat distribution (Pateyjohns, Brinkworth et al. 2006). The advantages of bio-impedance analysis include its ease of use, safety, portability, relatively low cost and require minimal participant participation. However this technique should not be used in subjects with a pacemaker in situ.

**Insulin sensitivity**

**Homeostatic model assessment (HOMA)**

HOMA is a method for assessing β cell function and insulin sensitivity/insulin resistance from basal blood glucose and insulin concentrations. The HOMA model is derived from a computer based mathematical assessment of the interaction between pancreatic islet function and insulin resistance. The output is calibrated to normal β cell function of 100% and normal insulin resistance of 1(Wallace, Levy et al. 2004). Experimental data from humans and animals form the basis of predictions used in the model.

The uptake and output of glucose from the liver are modelled to depend on the glucose and insulin levels. In HOMA insulin half-life is defined as 3.8 minutes with an additional slower component(Bergman and Bucolo 1974). The glucose uptake in fat and muscle is determined by the insulin concentration(Matthews, Rudenski et al. 1985). In humans approximately half of the basal glucose turnover is to the central nervous system, which is a glucose dependent process (Rowe, Maxwell et al. 1959). Glucose uptake by the muscle and fat are dependent on both glucose and insulin concentrations(Berger, Hagg et al. 1975).

**HOMA2: the updated HOMA model**

The currently used version HOMA2 has nonlinear solutions and accounts for variations in hepatic and peripheral glucose resistance. In addition adjustments are made to allow for an increase in insulin secretion if the plasma glucose is > 10 mmol/l. To further improve accuracy the effect of glycosuria due to hyperglycaemia is also taken into account(Rudenski, Matthews et al. 1991). The mean of 3 samples taken at 5 minute intervals is better than a single sample, but this is often impractical. In larger datasets analysis of a single sample give s comparable results to the mean of three samples(Wallace, Levy et al. 2004). As haemolysis degrades insulin, careful handling of the samples is essential for accurate results.

### Caveats for using HOMA

HOMA can be used to assess insulin sensitivity in subjects treated with exogenous insulin, but it should be ensured that blood sampling is done when glucose and insulin concentrations are in a steady state(Wallace, Levy et al. 2004). It is not appropriate to use HOMA to calculate β cell function in subjects treated with exogenous insulin. As HOMA is a measure of basal insulin sensitivity and β cell function, it does not give information about the stimulated state (Wallace, Levy et al. 2004).

## Incretins

### Overview of incretins

It was noted that the insulin response following oral administration of glucose was two to three times higher, when compared with the insulin response after intravenous glucose (Holst and Orskov 2001). This phenomenon is due to the 'incretin effect' mediated by the gut derived hormones GLP-1 and GIP (Nauck, Schmidt et al. 1989, Nauck, Bartels et al. 1993). The magnitude of incretin effect depends on the glucose load ingested, thereby keeping blood glucose levels stable (Nauck, Homberger et al. 1986). Both hormones have short half-lives and are cleared from circulation by the enzyme DPP4(Meier, Nauck et al. 2004).

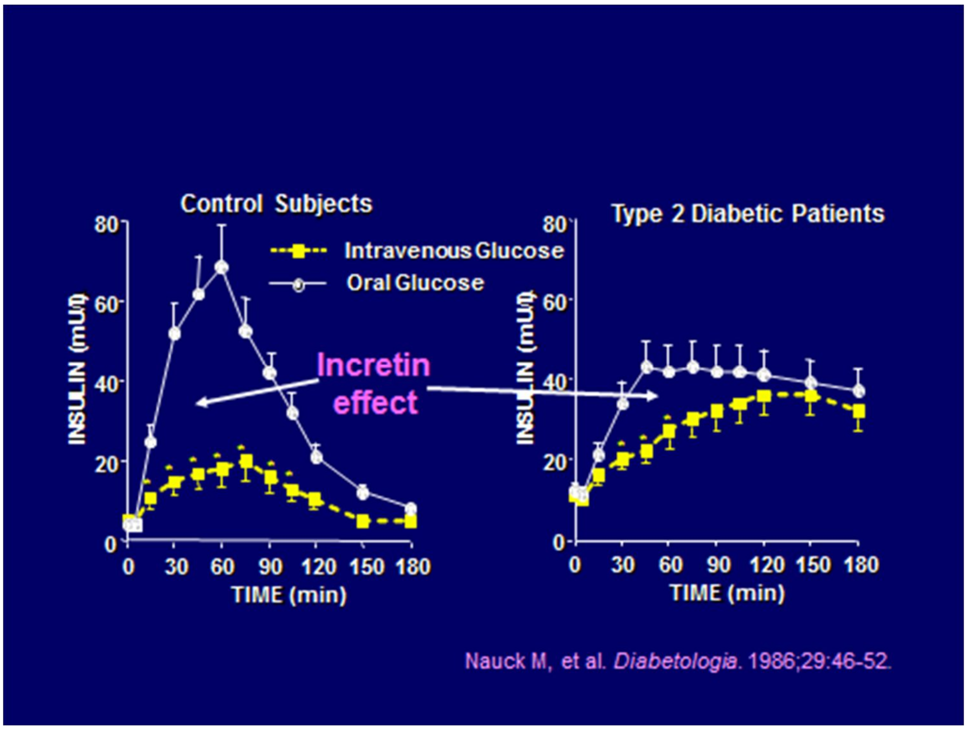



Figure 1.4: GRAPH DEPICTING THE INCRETIN EFFECT. The area under the curve for insulin secretion is smaller in subjects with type 2 diabetes when compared with controls.

**Incretin based therapies**

Experimental studies on humans have shown that the GLP-1 response to a glucose load is reduced in patients with type 2 diabetes, whereas the GIP response is relatively preserved (Vilsbøll, Krarup et al. 2001). This impaired secretion of GLP-1 might contribute towards inappropriate insulin secretion in type 2 diabetes. Treatments based on the incretin molecule GLP-1 are recent additions to medical management of type 2 diabetes (Lovshin and Drucker 2009).

The inhibitors of DPP4 enzyme (sitagliptin, vildagliptin, saxagliptin and linagliptin) reduce the enzyme activity by up to 80% and prolong the action of endogenous GLP-1. This generally produces a modest and transient rise in post prandial blood concentrations of GLP-1 (Ahrén, Landin-Olsson et al. 2004, Herman, Stevens et al. 2005). The analogues of GLP-1 (exenatide, liraglutide and lixisenatide) are resistant to enzymatic degradation by DPP4 enzyme. Following parenteral administration, these drugs achieve sustained pharmacological blood concentrations, hence are more effective than DPP4 inhibitors in achieving glycaemic control (Drucker and Nauck 2006).

As GLP-1 and its analogues induce insulin secretion from the pancreatic β cells only when blood glucose levels are raised. Therefore, when used as monotherapy or in combination with metformin, the chances of inducing hypoglycaemia are minimal. The GLP-1 analogues and DPP4 inhibitors have rapidly become an established component of the management of type 2 diabetes, due to their weight neutral/weight loss effects and due to their lower propensity to cause hypoglycaemia (Lovshin and Drucker 2009).

## Effects of GLP-1 beyond glycaemic control

### Distribution of the GLP-1 receptor

The GLP-1 receptor has been found in a number of human tissues and organs, such as brain, heart, breast, kidney, endothelium, lung and intestine (Körner, Stöckli et al. 2007). The receptor is also expressed in certain tumours such as pheochromocytomas. Although the principal action of GLP-1 is to regulate insulin secretion, it also has a number of effects in various organ systems. By extension, the actions of long acting GLP-1 analogues are also manifested in various tissues and organs.

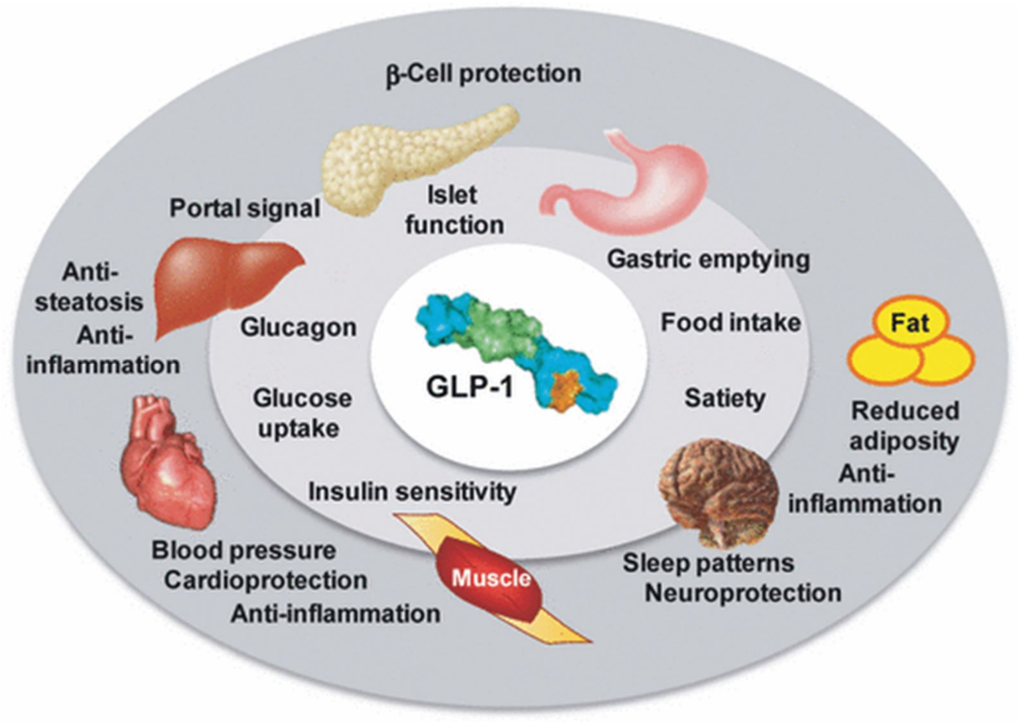



Figure 1.5: CARTOON DEPICTING ACTION OF GLP-1 ON VARIOUS ORGAN SYSTEMS. The chief actions of incretin hormones are to induce insulin secretion, delay gastric emptying and induce satiety.

## GLP-1 effects on blood pressure

Liraglutide Effect and Action in Diabetes (LEAD) are a series of large randomised controlled trials comparing the effect of the long acting GLP-1 analogue liraglutide, with various treatments for type 2 diabetes (Parks and Rosebraugh 2010). In these trials a modest (2 to 6 mmHg) reduction in systolic blood pressure was noted in patients treated with liraglutide (Garber, Henry et al. 2009, Nauck, Frid et al. 2009). There was no significant change in diastolic blood pressure. It is possible that some of the improvement in systolic blood pressure was due to the significant weight loss seen in subjects on liraglutide. However, in at least one of the studies, the change in blood pressure was seen before any weight loss was noted, suggesting an independent effect of the drug (Russell-Jones, Vaag et al. 2009). In a clinical trial setting, patients treated with exenatide also showed a trend towards lower blood pressures (Gill, Hoogwerf et al. 2010).

Evidence from animal models suggests that the blood pressure lowering effect of the incretins is due to the action on central and peripheral neural pathways (Barragán, Eng et al. 1999). Part of the blood pressure lowering effect of GLP-1 might be due to its diuretic and natriuretic actions (Crajoinas, Oricchio et al. 2011). Experiments using mice hearts have demonstrated secretion of atrial natriuretic peptide (ANP) following activation of the GLP-1 receptor in the atrium (Kim, Platt et al. 2013). Hence a role for gut-heart axis in regulating blood pressure cannot be ruled out.

## GLP-1 effects on endothelium

Following treatment with exenatide, monocytes of rats showed a lower propensity to adhere to the endothelium of isolated aortae (Arakawa, Mita et al. 2010). In vitro studies have demonstrated the expression of GLP-1 receptor on the vascular smooth muscle cells of humans (Goto, Nomiyama et al. 2011). Treatment with exenatide significantly reduced the proliferation of cultured smooth muscle cells that was induced by platelet derived growth factor (Goto, Nomiyama et al. 2011).

In vitro studies have demonstrated that liraglutide attenuated the induction of plasminogen activator inhibitor type-1 and vascular adhesion molecules in human vascular endothelial cells (Gaspari, Liu et al. 2011). In vivo studies on mice showed a significant improvement in endothelial dysfunction following treatment with liraglutide (Gaspari, Liu et al. 2011). Advanced glycation end products play an important role in the development of microvascular complications of diabetes. GLP-1 inhibits the expression of the receptor for advanced glycation end products on isolated human umbilical vein endothelial cells in a dose dependent manner (Ishibashi, Matsui et al. 2010). Further in vitro studies have shown that liraglutide exerts anti-oxidant and anti-inflammatory effects on endothelial cells and up regulates protective anti-oxidative enzymes (Shiraki, Oyama et al. 2012).

The effect of exenatide on post-prandial endothelial dysfunction was explored in a study, involving 28 subjects with impaired glucose tolerance or recent onset type 2 diabetes (Koska, Schwartz et al. 2010). In a double-blinded, randomised cross-over design the subjects were given a single injection of exenatide or placebo before a high fat meal. The post prandial endothelial function was significantly higher after exenatide. A large proportion (64%) of this effect was attributed to the reduction in post prandial triglyceride levels following exenatide.

These studies suggest that GLP-1 might have a significant role in modulating the course of vascular complications of diabetes. Large scale human studies have not yet been conducted to conclusively answer this question.

**GLP-1 effects on cardiovascular system**

In a clinical study, 10 patients and 11 matched controls were treated with a 72 hour infusion of GLP-1 or placebo. All the subjects had an ejection fraction <40% following acute myocardial infarction and were treated with angioplasty. The GLP-1 treated patients had a significant improvement in the mean ejection fraction from 29% to 39% ($p<0.01$). The improvements in the ejection fractions of GLP-1 treated subjects were evident in patients with and in subjects with normal glucose homeostasis (Nikolaidis, Mankad et al. 2004). In another randomized controlled trial, 172 patients with acute myocardial infarction were randomised to receive either exenatide or placebo. Even though the mean infarct size was smaller in the exenatide treated group, there was no difference in the ventricular function or 30 day event rate (Lønborg, Vejlstrup et al. 2012). In patients with NYHA (New York Heart Association) heart failure grades 3 or 4, infusion of GLP-1 significantly improved left ventricular ejection fraction, VO2 max and six minute walk distance when compared with placebo (Sokos, Nikolaidis et al. 2006). The exact mechanisms mediating the effects of GLP-1 and its analogues on the heart are still unclear.

## GLP-1 and the nervous system

Part of the weight reducing effects of GLP-1 analogues is attributed to induction of satiety by acting on the central nervous system. In an experimental study on human volunteers, positron emission tomography was employed to detect changes in blood flow to regions of brain. It was noted that blood levels of GLP-1 were associated with increased blood flow to the left dorsolateral prefrontal cortex and the hypothalamus, which are involved in satiety and feeding responses (Pannacciulli, Le et al. 2007). There is ample evidence that the increasing incidence of diabetes is due to the global rise in the rates of obesity (Shaw, Sicree et al. 2010, Vinciguerra, Baratta et al. 2013). The weight reducing effect of GLP-1 analogues makes them an attractive option for the management of type 2 diabetes as most other anti-hyperglycaemic drugs are either weight neutral or induce weight gain.

In vivo animal studies suggest that GLP-1 might have neuroprotective properties. Administration of vildagliptin to rats slowed nerve fibre loss, when compared with untreated rats. This suggests a possible beneficial effect of incretin based therapies in diabetic neuropathy (Jin, Liu et al. 2009). In a rodent model of Parkinson’s disease, exenatide promoted neurogenesis and restored dopaminergic imbalance in the basal ganglia (Bertilsson, Patrone et al. 2008).

**Renal effects of GLP-1**

Experiments on obese humans have shown that short term infusion of GLP-1 enhances renal sodium loss and reduces hyperfiltration. This suggests a possible action on the proximal tubule, which is the major site of sodium reabsorption (Gutzwiller, Tschopp et al. 2004). There is evidence suggesting that GLP-1 acts on the sodium/hydrogen exchanger on the proximal tubule (Crajoinas, Oricchio et al. 2011). However, it is not known whether this effect persists with continued administration of GLP-1 analogues. DPP4 inhibitors have also been shown to have natriuretic properties and hence might be a treatment option for salt-sensitive hypertension (Tanaka, Nangaku et al. 2011).

In vitro studies on mesangial cells indicate that GLP-1 might protect the renal cells from inflammatory damage due to advanced glycation end products (Ishibashi, Nishino et al. 2011). Administration of exenatide to rats for 8 weeks reduced albuminuria and glomerular hyperfiltration. In addition structural changes such as amelioration of glomerular hypertrophy were also noted. These effects were not linked to changes in glycaemia or blood pressure and were possibly mediated by modulation of pro-inflammatory cytokines (Kodera, Shikata et al. 2011).

These findings are very exciting in the context of management of diabetic nephropathy. Current management of diabetic nephropathy is based on optimizing glycaemic control and treatment of blood pressure.

On-going research is focussed on discovering drugs acting on inflammatory pathways involved in the pathogenesis of nephropathy, therefore, the GLP-1 based drugs might present an interesting option.

**GLP-1 and gastro-intestinal tract**

In a human study, the gastric volumes were evaluated by single photon emission tomography following intravenous infusion of GLP-1. When compared to controls the gastric volumes were higher in subjects who had GLP-1 (Delgado-Aros, Kim et al. 2002). Slowing of gastric emptying is one of the proposed mechanisms responsible for the glucose lowering effect (Marathe, Rayner et al. 2011). However continuous activation of the GLP-1 receptor on the stomach might result in reduced responsiveness. Indeed, the changes in gastric motility induced by long acting version of exenatide are smaller than the reductions achieved by twice daily injections of exenatide (Drucker, Buse et al. 2008).

In clinical practice, the most common adverse effects of GLP-1 analogues are related to the upper gastrointestinal tract and are thought to be due to gastric distension. Up to a quarter of patients started on liraglutide report nausea and vomiting, however symptoms persist in only 5-10% of patients after 4 weeks of continued therapy (Garber, Henry et al. 2009). In about 5% of patients, these adverse effects might be severe, requiring discontinuation of the drug (Garber, Henry et al. 2009, Marre, Shaw et al. 2009, Nauck, Frid et al. 2009).

**GLP-1 and the exocrine pancreas**

Recent publications have initiated a debate about the effects of GLP-1 on the exocrine pancreas, specifically about the risks of pancreatic cancer and pancreatitis (Barnett and O'Hare 2013). Results of a large retrospective study suggested that treatment with exenatide or sitagliptin might increase the risk of pancreatitis (Elashoff, Matveyenko et al. 2011). However it should be noted that the study did not control for confounding factors such as obesity, alcohol consumption and smoking. Another population based case-control study which was designed to control for the above mentioned factors indicated a twofold increased risk of pancreatitis with these drugs (Singh, Chang et al. 2013). Pooled analyses of multiple studies involving a number of GLP-1 analogues and DPP4 inhibitors did not show any increased risk of pancreatitis with these classes of drugs (Shyangdan, Royle et al. 2011, Alves, Batel-Marques et al. 2012, Engel, Round et al. 2013, Monami, Dicembrini et al. 2013).

In toxicology studies mice were treated with vildagliptin for two years with doses up to 200 times the recommended equivalent doses in humans. Dysplastic changes were seen in the pancreata of mice at such high doses (Busch, Hoffmann et al. 2013). The relevance of this finding to clinical management using DPP4 inhibitors is questionable.

It is unclear whether there is a link between the uses of GLP-1 based therapies and diseases of the exocrine pancreas. However it seems prudent that these classes of drugs should be used with caution in patients at risk of pancreatic disease due to other factors.

### GLP-1 and pancreatic β cell function

Administration of exenatide to rats was associated with an increased first phase glucose-stimulated insulin secretion after a period of 8 weeks. Interestingly an expansion of β cell mass was also noted, which was achieved by an increase in proliferation and a reduction in apoptosis (Tourrel, Bailbé et al. 2001). Similarly rats treated with liraglutide also showed improvement in β cell number and function (Shimoda, Kanda et al. 2011). Studies on obese humans with type 2 diabetes suggest an improvement in β cell function under conditions of glucose-insulin homeostasis (Preumont, Hermans et al. 2010). There is no evidence of β cell regeneration in humans.

### GLP-1 effects on metabolic rate

Studies on animal models suggests that GLP-1 might be involved in the control of adipokine secretion and energy expenditure (Hansotia, Maida et al. 2007). Blocking the GLP-1 receptors in the brains of mice induced hyperinsulinemia, insulin resistance and decreased energy expenditure. This suggests that GLP-1 might play an important role in energy homeostasis (Knauf, Cani et al. 2008). It has been proposed that GLP-1 is involved in post-prandial energy expenditure, mediated by the lower brain stem and sympathoadrenal system of rats (Osaka, Endo et al. 2005).

However these results have not been replicated in human studies. One experimental study on healthy human volunteers found a positive correlation between resting energy expenditure and fasting GLP-1 levels (Pannacciulli, Bunt et al. 2006). These results are difficult to interpret because GLP-1 is predominantly secreted in response to food and hyperglycaemia. Two human studies involving obese and normal weight subjects did not show any change in energy expenditure after treatment with liraglutide (Harder, Nielsen et al. 2004, Horowitz, Flint et al. 2012).

**GLP-1 effects on body composition**

In a study involving subjects with mild obesity, short term treatment with liraglutide showed an improvement in visceral adiposity as measured by bio-electrical impedance analysis (Inoue, Maeda et al. 2011). A post-hoc analysis of LEAD-2 and LEAD-3 trials confirmed these results. Significant reductions in total fat mass and percentage of body fat were noted. In the subgroup of patients on liraglutide 1.8 mg/day, a significant change in liver-to-spleen attenuation ratio was seen, indicating a reduction in hepatic steatosis (Jendle, Nauck et al. 2009). Treatment with exenatide for 1 year significantly reduced body weight by 6%. Interestingly the reductions in total body fat and trunkal fat mass were 11% and 15% respectively. In addition, exenatide increased total adiponectin by 12%, independent of changes in total body weight or body fat mass (Bunck, Cornér et al. 2010). These results indicate that GLP-1 analogues might preferentially reduce adiposity in obese subjects. All the above mentioned studies were done on human subjects who were either overweight or had a mild degree of obesity. Studies have not yet explored the effect of these drugs on subjects with morbid obesity.

## Hypothesis

The microvascular complications of diabetes are responsible for much of the morbidity due to the disease. There is good evidence that the risks of diabetic nephropathy and retinopathy are mitigated by achieving tight glycaemic control and appropriate management of blood pressure. Recent evidence suggests that inflammation and endothelial dysfunction play a pivotal role in the development and progression of these complications. In vitro studies indicate that the recently licenced GLP-1 analogues have anti-inflammatory properties and improve endothelial function. Indeed, work on animal models suggests that GLP-1 analogues slow the onset and progression of diabetic nephropathy and improve outcomes in diabetic retinopathy. Hence, I hypothesised that the GLP-1 analogue, liraglutide might replicate this beneficial effect in humans.

Previous human studies have shown an improvement in body composition and in markers of cardiovascular risk following treatment with liraglutide. Almost all studies have been performed on subjects who were classed as overweight or had a mild degree of obesity. Clinical studies have not included patients with morbid obesity who have the highest levels of cardiovascular risk. Hence, I investigated whether liraglutide modulates the cardiovascular risk of patients with type 2 diabetes and morbid obesity.

## Aims

To investigate whether treatment with liraglutide

1. slows the progression of microalbuminuria in subjects with established abnormal urinary albumin excretion
2. influences the rate of progression of urinary albumin excretion in normoalbuminuric subjects
3. changes the course of diabetic retinopathy in patients with type 2 diabetes
4. modulates cardiovascular risk and body composition in morbidly obese patients

# CHAPTER 2

# METHODS

## Ethical approval

This research project was undertaken as a sub-study of the 'Investigation of metabolism in human participants' project that is on-going at University hospital of Coventry and Warwickshire. Formal ethical approval has been granted for this study by the - Birmingham, East, North and Solihull regional ethics committee (REC reference number: 11/H1206/3).

## Retrospective Study

### Retrospective data collection

Retrospective data was collected from the clinical case records of patients with type 2 diabetes treated at University hospital of Coventry and Warwickshire NHS trust. Case notes of all the patients who treated with liraglutide for at least 6 months were requested for data collection. We collected baseline anthropometric measurements prior to starting treatment with Liraglutide and follow up data at 6 and 12 months. The laboratory results of these subjects at baseline, 6 and 12 months were collected from the online Clinical Results Reporting System. Baseline laboratory tests were defined as tests performed not more than 4 weeks before commencement of treatment. Follow up laboratory results were included if they were performed within a 4 week window period 6 months and 12 months after treatment was started . Patients who were previously treated with any other GLP-1 analogue were excluded from our study.

Inclusion criteria

1. All patients with type 2 diabetes treated with liraglutide at University hospital of Coventry and Warwickshire

Exclusion criteria

1. Age less than 18 years or more than 80 years.
2. Previous treatment with GLP-1 analogues.
3. No hospital record of baseline and 6 month post treatment anthropometric and laboratory data.

A group of control subjects, who were not treated with liraglutide were identified from the DIAMOND database system at the University hospital of Coventry and Warwickshire NHS trust. The case records of these patients were requested for data collection. Cases and controls were matched for gender, age, duration of diabetes, baseline BMI, baseline HbA1c, baseline systolic blood pressure, baseline diastolic blood pressure, baseline total cholesterol and baseline urinary albumin/creatinine ratio. All cases and controls were treated with an ACE-I/ARB for blood pressure and other anti-hypertensive drugs if appropriate.

Inclusion criteria

1. All subjects with type 2 diabetes on DIAMOND database at University hospital of Coventry and Warwickshire.

Exclusion criteria

1. Treatment with liraglutide or other GLP-1 analogues.
2. No 6 monthly record of anthropometric and laboratory data.
3. No record of annual retinal screening data.

**Statistical analysis**

Statistical analysis was done using SPSS version 21. Independent samples ‘t’ test was employed to detect any baseline differences in continuous variables using ‘treatment with liraglutide’ as the grouping variable. Levene’s test was employed to test for equality of variances in the two groups. Students’s or Welch’s ‘t’ test was chosen based on the homogeneity of variance. Chi-square test for independence was employed for baseline categorical variables.

I calculated the change in anthropometric measurements and laboratory data 6 months after treatment for the liraglutide treatment group. Similarly, changes in the same parameters at 6 months were calculated for the controls. Change in albumin/creatinine ration at 6 months was defined as the primary outcome variable. Changes in BMI, HbA1c, total cholesterol, systolic and diastolic blood pressures were defined as secondary outcome variables. Independent samples ‘t’ test was employed to detect any significant change between groups with ‘treatment with liraglutide’ as grouping variable. A ‘p’ value less than 0.05 was defined as indicating statistical significance.

12 month follow up data was available for a subgroup of patients who continued treatment with liraglutide. This subgroup was again matched with an

appropriate control group and statistical analysis was done as mentioned previously.

**Criteria for normal and abnormal urinary ACR**

As suggested by the NICE guidelines, patients were classed as having microalbuminuria if urinary Albumin/Creatinine ratio $\geq$ 2.5 mg/mmol for men and $\geq$ 3.5 mg/mmol for women. Data from cases and controls with established microalbuminuria were compared to detect the effect of liraglutide on progression of nephropathy. Data from patients with albumin excretion in urine but not diagnostic of microalbuminuria was analysed to detect the effect of liraglutide on the onset of diabetic nephropathy.

**Data collection for retinopathy study**

For our retrospective retinopathy study, cases and controls were identified as mentioned above. Patients with diabetes in the catchment area of University hospital of Coventry and Warwickshire NHS trust are offered an annual retinal photograph as part of a national screening program and the retinopathy grades are recorded in the 'OPTOMIZE' database. The retinal photographs are graded as per nationally agreed criteria for retinopathy (R0-no retinopathy, R1-background retinopathy, R2-pre proliferative retinopathy, R3-proliferative retinopathy), maculopathy (M0-no maculopathy, M1-presence of maculopathy) and previous photocoagulation (P0-no photocoagulation, P1-previous photocoagulation). From the database we recorded the grades for retinopathy, maculopathy and previous photocoagulation in addition to visual acuity scores for all patients treated with liraglutide.

Subjects were included in the study if they had a retinal screening before starting liraglutide and the interval between this initial retinal screening and commencement of liraglutide was not more than 6 months. We recorded the R, M and P grades of these subjects after 12 months if they continued treatment with liraglutide. Subjects who had stopped taking liraglutide before the second retinal photograph were excluded. For the control group we recorded two consecutive retinal grades performed at least 12 months apart.

## Prospective study

### Patient recruitment for prospective study

Patients attending the WISDEM centre at University hospital of Coventry and Warwickshire were invited to take part in the on-going human metabolism research projects in the hospital. Patients who expressed interest were contacted by me and my colleagues to discuss the study in more detail, following which a copy of the information sheet was provided. The subjects were asked to reflect for at least 24 hours before consenting to participate in the study. All participants were requested to give a formal signed consent prior to involvement in the research project.

Inclusion criteria

1. All subjects with type 2 diabetes commencing treatment with liraglutide form 01/11/2011 to 31/10/2012 at University hospital of Coventry.

Exclusion criteria

1. Subjects less than 18 years or older than 80 years.
2. Previous treatment with GLP-1 analogues.
3. Unwilling to participate or unable to consent.
4. Unable to attend follow up visit after 3 months.

We prospectively studied a group of 20 subjects with type 2 diabetes treated with liraglutide for at least 3 months at the discretion of their physician. All 20 of the subjects had hypertension and were treated with an ACE-1/ARB and other anti-hypertensive medications if appropriate. In this group 11 (7 males and 4 females) patients had established microalbuminuria at baseline and 9 (5 males and 4 females) patients had albumin excretion in urine, but not diagnostic of microalbuminuria. Fasted blood and urine samples were collected from all subjects before starting liraglutide and the same was repeated after 3 months of treatment. In addition anthropometric data, clinical biochemistry and body composition analysis were done on these patients before starting treatment and 3 months after treatment. Assays for biomarkers of diabetic nephropathy were performed on the plasma samples before and after treatment.

**Body composition analysis using InBody 720 analyser**

The body composition analyser, InBody 720 is accurate for all body types and uses a diverse range of frequencies from 1 kHz to 1 mHz to estimate body composition. The following precautions were taken in all our subjects before body composition analysis was performed to standardize the results

1. No significant exercise or exertion prior to analysis
2. All subjects were analysed after an overnight fast
3. Measurements were done under normal room temperature (20-25 ◦C)
4. Measurements were performed after urination and defecation

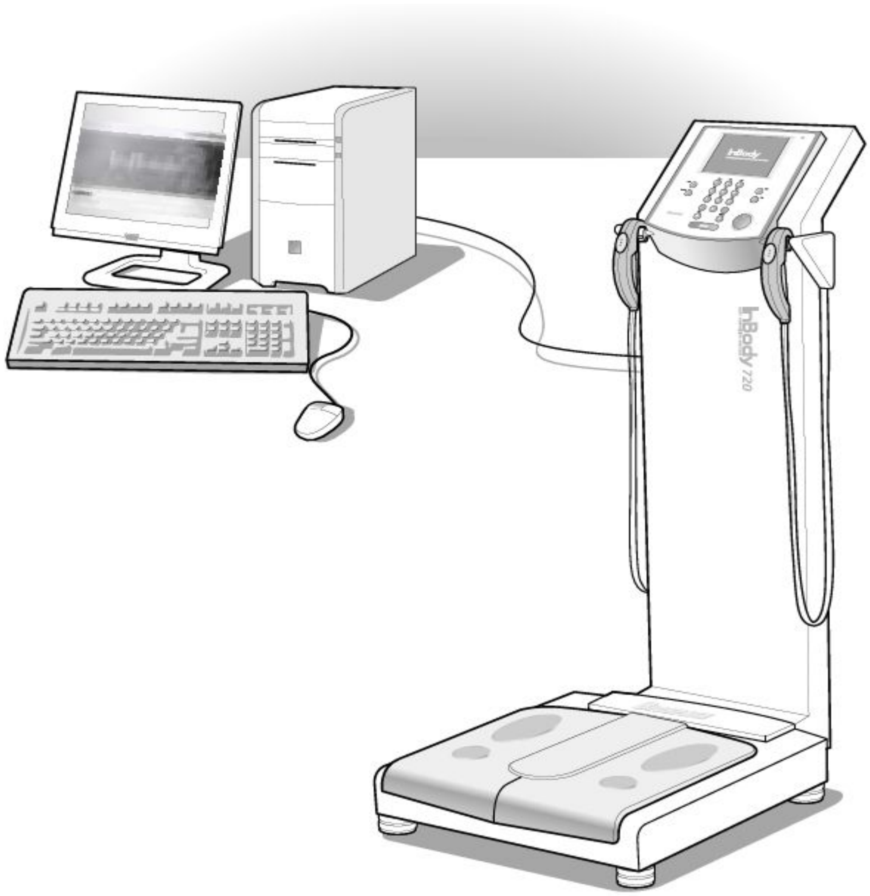



Figure 2.1: CARTOON OF INBODY720 BODY COMPOSITION ANALYSER

After recording the age and gender on the analyser, subjects were invited to step on the foot electrode, with their heels on the circular foot electrode and forefoot in contact with the fore foot electrode. Subjects were instructed to hold the hand electrodes with their thumbs on the thumb electrode and stand comfortably during the test forming a 15 degree angle between the arms and the trunk. The results were stored on the computer connected to the analyser for future analysis. The InBody uses an 8-point tactile method, which separates the current and voltage starting point. Thus measurement always begins at a fixed point in the wrists and ankles, providing high reproducibility and correct body impedance measurement because the contact resistance from the skin is removed.

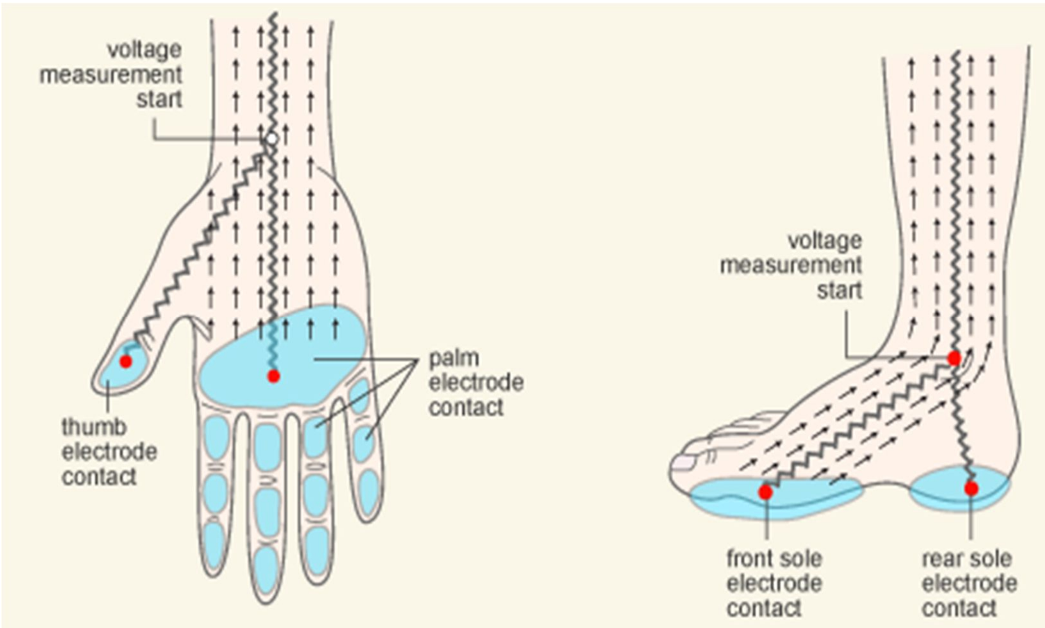



Figure 2.2: CARTOON DEPICTING ELECTRODE CONTACTS AND MECHANISMS OF BIO-IMPEDANCE ANALYSIS. The 8 point analyser involves 2 contacts in each of the four limbs facilitating accurate estimations of body composition.

## Bio-Plex assay

### Principle

I employed the luminex based Bio-Plex assays to measure the serum concentrations of MCP-1, VEGF, sICAM-1 and sVCAM-1. The Bio-Plex multiplex assays are immunoassays formatted on magnetic beads. The assay principle is similar to a sandwich ELISA. Capture antibodies against the molecule of interest are covalently coupled to the beads, which react with human plasma samples. After multiple washes to remove unbound protein, a biotinated detection antibody is added to create a sandwich complex. Phycoerythrin bound to streptavidin is used as the fluorescent indicator in the final detection complex.

Data from the reactions are acquired using the Luminex based Bio-Plex system. When the assay suspension is drawn into the reader, a red (635 nm) laser illuminates each bead to provide bead classification and assay identification. Similarly a green (532 nm) laser induces the phycoerythrin to generate a reporter signal which is detected by a photomultiplier tube. The concentration of analyte bound to each bead is proportional to the median fluorescence intensity of the reporter signal.

Multiple independent studies have noted a mean inter assay coefficient of variability of 10-14% for multiplexed bead based assays for cytokine detection (dupont, Wang et al. 2005, Dossus, Becker et al. 2009). Intra-assay precision of duplicate wells for ICAM-1 and VCAM-1 is less than 10% (Stanford). The Bio-Plex assay for MCP-1 was found to detect the molecule over a concentration range of 1.45-23735 pg/ml, therefore superior to ELISA which had a detection range of 7.8-500 pg/ml. The mean coefficient of variability of Bio-Plex MCP-1 assay was 7.02% in one study(Christianssona, Mustjokib et al. 2014). The Bio-Plex VEGF assay has a detection range of 1.79-29354 pg/ml and mean coefficient of variability of 5.01% (Christianssona, Mustjokib et al. 2014)

**Preparation and assay technique**

The Bio-Plex system was started up and calibrated as per the manufacturer's protocol. Lyophilized standard was reconstituted with 500 µl of standard diluent, which was serially diluted to produce an eight-point standard curve with a fourfold dilution between each point. For the human MCP-1 and human VEGF assays, plasma samples were diluted fourfold with sample diluent. For the human ICAM-1 and human VCAM-1 assays, the plasma samples were diluted to 1:100 with sample diluent. Appropriate volumes of coupled beads were calculated using the worksheet provided and diluted in assay buffer. Using a multichannel pipette 50 µl of the diluted coupled beads was added to each well of a 96 well plate. After adding 50 µl of the diluted standards, blanks and plasma samples as appropriate to each well, the plate was incubated on a shaker at room temperature for 30 minutes.

After washing three times with wash buffer, the prepared detection antibody was added to each well and incubated on a shaker for 30 minutes. After washing three times, the prepared streptavidin- phycoerythrin was added. Following further 30 minute incubation and washing 125 µl of assay buffer was added to each well.

The plate was formatted in the Bio-plex software to identify the wells with standards, blanks and plasma samples. Data acquisition was set to 100 beads per region for MCP-1 and VEGF assays. For ICAM-1 and VCAM-1 assays, the data acquisition was set to 50 beads per region. For all the assays sample size was set to 50 μl and DD gates set to 5000 (low) and 25000 (high). Data acquired from the plate reader was analysed as described in detail in results section.

## Assay for hsCRP

### Principle

The Tina-quant immunoturbidimetric assay employed to measure hsCRP in human plasma samples on Roche analysers. The assay is based on the principle of particle enhanced immunological agglutination. Anti-CRP antibodies coupled to latex microparticles react with antigen in the plasma sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically.

Independent verification by FDA noted that inter-assay coefficient of variation ranged from 0.6% to 1.3%, whereas inter-assay coefficient of variability ranged from 2.2% to 3.5 (FDA). The analytical sensitivity (lower detection limit) of the assay was 0.1 mg/L and functional sensitivity (limit of quantitation) was 0.3 mg/L (FDA). The assay was accurate even in the presence of haemolysis, icterus, paraproteins or lipemia.

### Assay technique

Reagent 1 for the assay is Tris(hydroxymethyl)-aminomethane buffer 16 mmol/l, pH 7.4 to form reagent 1. Reagent 2 comprises of latex particles coated with anti-CRP mouse monoclonal antibodies: 0.1%; glyceine buffer: 50 mmol/l, pH 8.0. Reagents 1 and 2 are added to human plasma to start the reaction. The Roche/Hitachi 902 analyser is caliberated with Preciset serum proteins, Cal 2. After appropriate incubation the assay is run on the analyser. The measurement range of hsCRP on this assay is 0.1 to 20 mg/l.

## Assay for insulin

### Principle

Immunoassay for quantification of insulin in human plasma was done using the Elecsys assay which employs two monoclonal antibodies that are specific for human insulin. This is a sandwich ELISA assay.

The Diabetes Research Unit at Cardiff University conducted an independent study on the various commercially available insulin assays in the UK. The coefficient of variability of the above mentioned assay ranged from 1.1% to 1.7%. Sensitivity of the assay was 1.4 pmol/L and the upper limit was 6945 pmol/L (Manley, Stratton et al. 2007).

Insulin from a 20 µl plasma sample, a biotinated monoclonal insulin-specific antibody, and a monoclonal insulin specific antibody labelled with a ruthenium complex form a sandwich complex. After addition of streptavidin coated micro particles, the complex becomes bound to the solid phase via interaction biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the micro particles are magnetically captured onto the surface of the electrode. Unbound substances are removed with ProCell. Application of electric current then induces chemiluminescent emission. This is quantified using a photo multiplier.

**Calibration**

The calibration is instrument specifically generated by a 2 point calibration and a master curve provided via the reagent barcode. Samples and calibrators were at ambient temperature of 20-25 centigrade before measurement. Due to possible evaporation effects, samples and calibrators were analysed within 2 hours.

**Reagents and technique**

M- Streptavidin coated micro particles 0.72 mg/ml

R1- Biotinated anti-insulin monoclonal antibody 1 mg/l, MES buffer 50 mmol/l, pH 6.0.

R2- Monoclonal mouse anti-insulin antibody labelled with ruthenium complex 1.75 mg/l, MES buffer 50 mmol/l, pH6.0.

Human plasma is incubated with R1 and subsequently with R2 for appropriate periods to form a sandwich complex. After a further incubation with streptavidin, the assay is read on the modular analytics E170 analyser. The measurement range for insulin on the assay is 1.39-6945 pmol/l.

# CHAPTER 3

## EFFECT OF LIRAGLUTIDE ON PROGRESSION OF URINARY ALBUMIN EXCRETION IN PATIENTS WITH TYPE 2 DIABETES MELLITUS AND ESTABLISHED MICROALBUMINURIA

## Introduction

Diabetes is currently the most common cause of end stage renal failure in the western world due to the rapid increase in the prevalence of diabetes, especially type 2 diabetes. In addition the life expectancy of patients with diabetes is increasing, hence the propensity to develop chronic complications such as diabetic nephropathy also increases (Molitch, DeFronzo et al. 2004). The majority of expenditure attributable to diabetes is not related to direct glycaemic control, but is mainly due to chronic complications and associated medical conditions(1998). Hence it is important to prevent development of chronic complications and to limit their progression if already present.

Microalbuminuria is the earliest clinical indicator and most reliable marker of diabetic nephropathy. It is defined as a urinary albumin/creatinine ratio (ACR) of >2.5 mg/mmol in men and >3.5 mg/mmol in women. Established microalbuminuria is confirmed if an abnormal urinary ACR is detected on at least two occasions within 3-4 months(National Collaborating Centre for Chronic 2008). About 25% of patients develop microalbuminuria after 10 years of type 2 diabetes, and a smaller proportion have a progressive increase in albuminuria(Adler, Stevens et al. 2003).

Good glycaemic control has been shown to slow the progression of microalbuminuria(Reichard, Nilsson et al. 1993). Tight control of blood pressure is another key element of management of patients with microalbuminuria (1998). Anti-hypertensives that block the renin-angiotensin system are thought to confer additional benefit over and above blood pressure reduction in improving renal outcomes in subjects with established microalbuminuria (Brenner, Cooper et al. 2001, Casas, Chua et al. 2005, Bilous, Chaturvedi et al. 2009). Other treatment options such as fenofibrate and dietary protein restriction have been proposed, but their effectiveness is questionable (Hansen, Tauber-Lassen et al. 2002, Ansquer, Foucher et al. 2005).

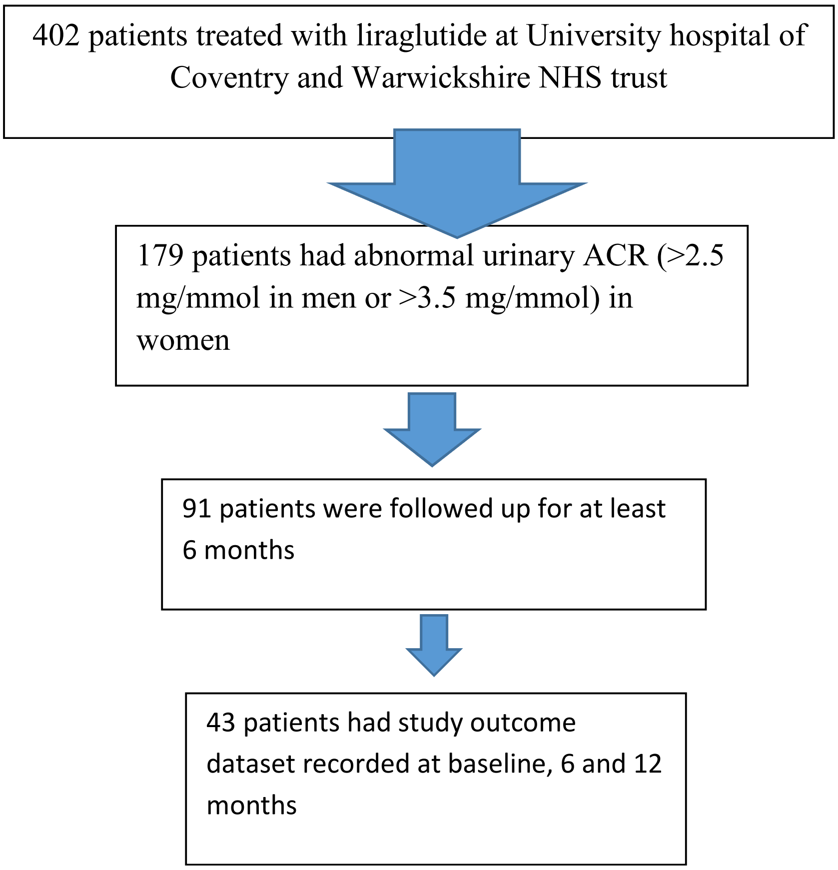
In animal models of diabetic nephropathy, GLP-1 based therapies have shown promising results in mitigation of diabetes related renal damage (Ishibashi, Nishino et al. 2011, Hendarto, Inoguchi et al. 2012). The effect is thought to be due to modulation of low grade inflammation and endothelial dysfunction which are associated with diabetic nephropathy.

**Aim**

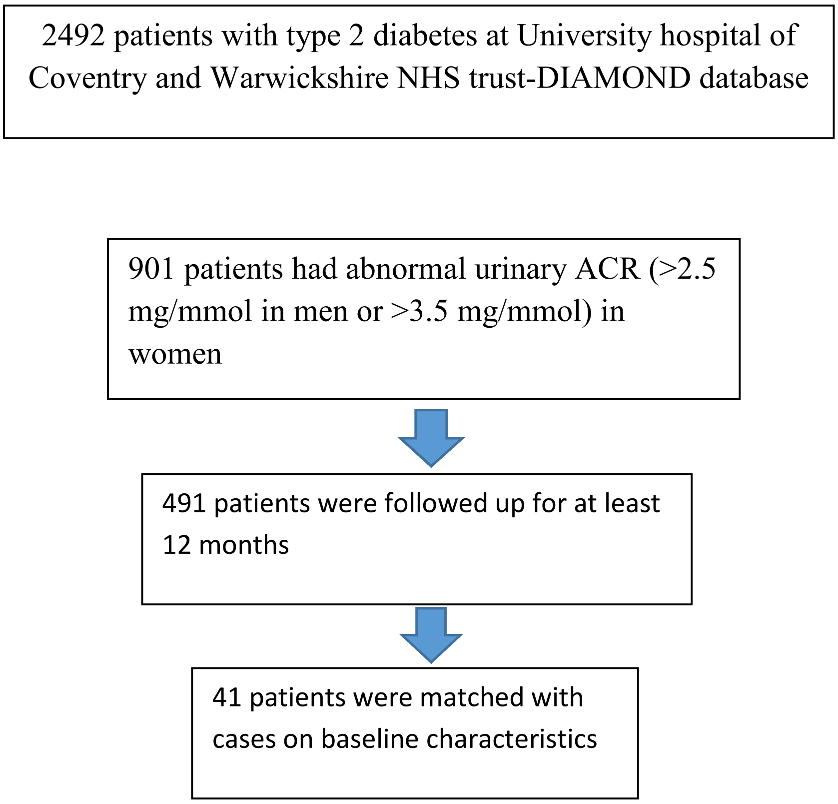
In this study I explored the effect of the GLP-1 analogue liraglutide on progression of urinary ACR in patients with established microalbuminuria.

**Methods**

In this single centre study, patients who were treated with liraglutide were identified form patient database of University hospital of Coventry and Warwickshire NHS trust. Clinical and anthropometric characteristics of these patients were collected form their case records at baseline, at 6 months and at 12 months if the treatment was continued. In particular the urinary ACR of the patients was recorded before treatment and 6 months after treatment.




A group of controls were identified from the DIAMOND database system and their characteristics were similarly recorded from the case records. Change in urinary ACR over 6 months and over 12 months were compared between the two groups using SPSS version 21 for statistical analysis.




Primary outcome

1. Changes in urinary albumin creatinine ratios after 6 and 12 months.

Secondary outcome

1. Changes in BMI, HbA1c, total cholesterol, systolic and diastolic blood pressures after 6 and 12 months

All patients attending the GLP-1 clinic to commence treatment with liraglutide were invited to participate in the prospective study. Details of patient recruitment are detailed in chapter 2-Methods. 24 patients had abnormal urinary albumin creatinine ratios, out of which, 11 patients agreed to participate in the study after.  Fasted blood samples were collected from these subjects before and 3 months after treatment with liraglutide. The plasma levels of ICAM-1, VCAM-1, MCP-1 and hsCRP were measured before and after treatment. Each serum sample was assayed for the molecule of interest in duplicate and the mean of the two values was used for statistical analysis. If the duplicates varied by >10%, that particular sample was excluded from the analysis. The details of these assays are discussed in chapter 2.

Primary outcomes

1. Changes in biomarkers of endothelial dysfunction (sICAM-1 an sVCAM-1) after 3 months of treatment with liraglutide.
2. Changes in biomarkers of inflammation (hsCRP and MCP-1) after 3 months of treatment with liraglutide.

Secondary outcomes

1. Changes in urinary albumin creatinine ratios after 3 months of treatment with liraglutide
2. Changes in BMI, HbA1c, total cholesterol, systolic and diastolic blood pressures after 3 months of treatment with liraglutide.

**Results**

In a retrospective cohort study we compared the baseline albumin/creatinine ratios of 43 patients (26 males, 17 females) with their albumin/creatinine ratios after 6 months of treatment with liraglutide. A matched control group of 41 patients with type 2 diabetes who were not treated with liraglutide were used as comparator. All cases and controls were treated with either an ACE-I or ARB for hypertension. Baseline characteristics of the subjects are shown on table 1.

| DOMAIN | LIRAGLUTIDE Mean (Standard Deviation) | CONTROL Mean (Standard Deviation) | Significance p= |
|---|---|---|---|
| **Number** | 43 | 41 | |
| **Males:Females (number)** | 26:17 | 24:17 | 0.85 (NS) |
| **Age (years)** | 59.93 ± 7.98 | 64.17 ± 11.68 | 0.11 (NS) |
| **Duration of Diabetes (years)** | 14.24 ± 8.25 | 14.62 ± 10.18 | 0.85 (NS) |
| **Weight (kg)** | 110.79 ± 26.77 | 106.81 ± 20.30 | 0.44 (NS) |
| **BMI (kg/m2)** | 37.36 ± 7.67 | 37.42 ± 5.74 | 0.96 (NS) |
| **HbA1c (mmol/mol)** | 71.14 ± 15.81 | 64.68 ± 15.06 | 0.09 (NS) |
| **Systolic Blood Pressure (mm Hg)** | 148.77 ± 18.58 | 143.61 ±16.29 | 0.18 (NS) |
| **Diastolic Blood Pressure (mm Hg)** | 78.40 ± 12.88 | 76.61 ± 11.31 | 0.50 (NS) |
| **Total Cholesterol (mmol/L)** | 4.05 ± 0.85 | 4.37 ± 1.20 | 0.16 (NS) |
| **Urinary Albumin/Creatinine (mg/mmol)** | 18.90 ± 23.33 | 14.62 ± 18.51 | 0.35 (NS) |

Table 3.1: BASELINE CHARACTERISTICS OF CASES AND CONTROLS WITH ABNORMAL ALBUMIN:CREATININE RATIO

Normality of distribution of the baseline variables were assessed by Kolmogorov-Smirov and Shapiro-Wilk tests using SPSS software (threshold for significance was 0.05). If the ‘p’ values for both tests were non-significant, these variables were considered normally distributed for the purpose of this study.

After 6 months there was a statistically significant reduction the urinary albumin excretion rates of subjects in both groups. The mean baseline ACR for all participants (liraglutide treated group and control group) was 16.81 mg/mmol, whereas the mean ACR after 6 months was 11.18 mg/mmol. The mean change in ACR was 5.63 mg/mmol (95% CI: 3.12 to 8.13, $p<0.01$).

Patients treated with liraglutide had greater reductions in their urinary albumin/creatinine ratios, but the results were not statistically significant. There was a significant reduction in HbA1c of 5.11 mmol/mol in the liraglutide group, but an increase of 3.75 mmol/mol in the control group. In addition there was a significant reduction in BMI of 1.02 kg/m$^2$ in the liraglutide group and in increase in BMI of 0.27 kg/m$^2$ in the control group. There was a trend towards statistically significant improvement in total cholesterol levels post treatment in the liraglutide group. Results are shown on table 2, figures 1 and 2.

| DOMAIN | LIRAGLUTIDE Mean difference (Standard error) | CONTROL Mean difference (Standard error) | Significance p= |
|---|---|---|---|
| Urinary Albumin/Creatinine (mg/mmol) | -6.90 ± 1.57 | -4.29 ± 1.98 | 0.30 (NS) |
| BMI (kg/m2) | -1.02 ± 0.27 | +0.27 ± 0.14 | 0.001 |
| HbA1c (mmol/mol) | -5.11 ± 2.21 | +3.75 ± 2.03 | 0.004 |
| Systolic Blood Pressure (mm Hg) | -4.92 ± 2.23 | -3.90 ± 2.86 | 0.77 (NS) |
| Diastolic Blood Pressure (mm Hg) | -0.19 ± 1.72 | -0.68 ± 1.90 | 0.84 (NS) |
| Total Cholesterol (mmol/L) | -0.20 ± 0.08 | +0.06 ± 0.11 | 0.06 (NS) |

Table 3.2: CHANGES IN PRIMARY AND SECONDARY ENDPOINTS FROM BASELINE TO 6 MONTH FOLLOW UP

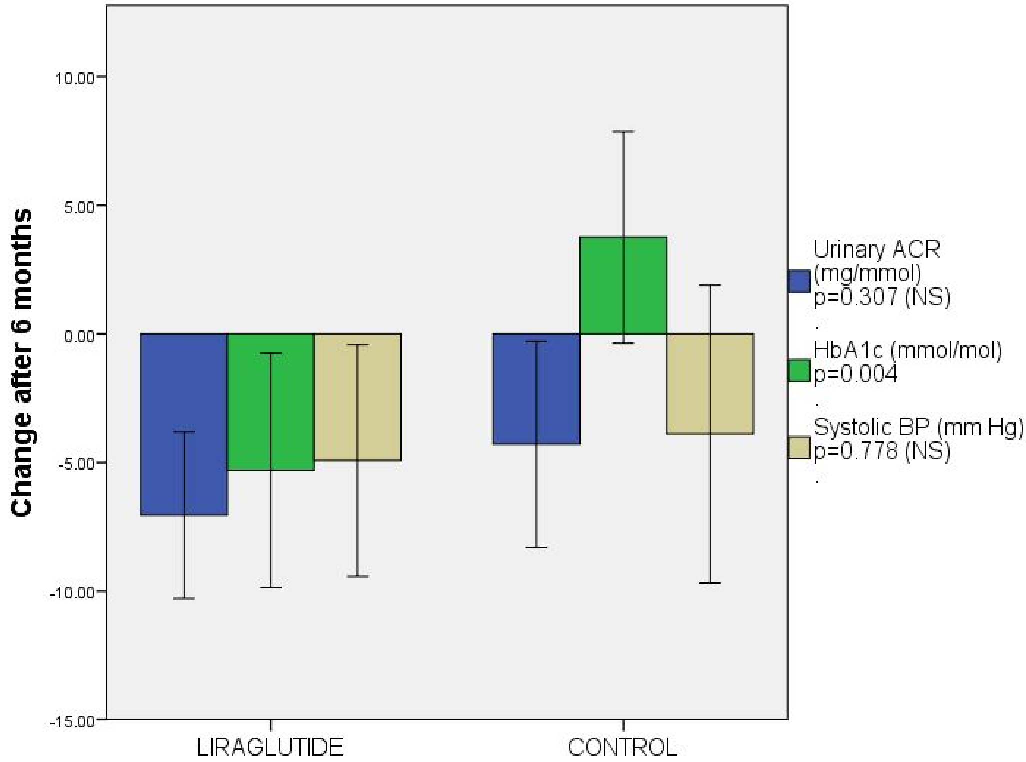



Figure 3.1: CHANGES IN ACR, HBA1C AND SYSTOLIC BP AFTER 6 MONTHS. Statistically significant improvements in HbA1c levels were noted in the liraglutide treated group, when compared with controls. However the differences in systolic blood pressures and urinary albumin/creatinine ratios were not significant.

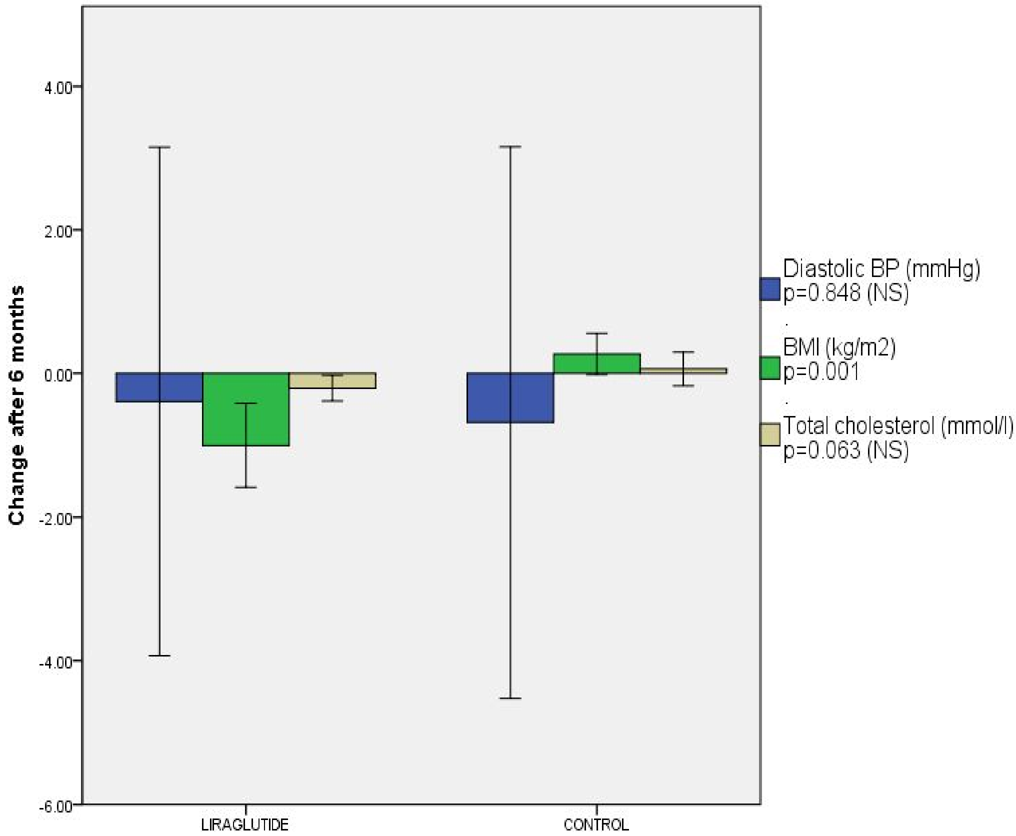



Figure 3.2: CHANGES IN DIASTOLIC BP, BMI AND TOTAL CHOLESTEROL AFTER 6 MONTHS. No statistically significant changes in diastolic blood pressures, BMI or total cholesterol levels were noted in the liraglutide treated group, when compared with controls.

Hierarchical linear regression was performed to identify the predictors of a change in urinary albumin excretion. Change in urinary albumin/creatinine ratio was the specified dependent variable. Age, duration of diabetes, baseline HbA1c, baseline urinary albumin/creatinine ratio, baseline systolic and diastolic blood pressures were entered as independent variables at step1. After controlling for these variables, change in HbA1c, changes in systolic and diastolic blood pressures were entered as independent variables in step 2. After step 2 the total variance explained by the model as a whole was 70%, $F$ (9, 69) =18.10, $p<0.001$. After controlling for all the above mentioned variables, treatment with liraglutide was entered as the independent variable at step 3. This did not explain any additional variance in urinary albumin/creatinine ratio. In the final model baseline urinary albumin/creatinine ratio and change in diastolic blood pressure were highly statistically significant. Baseline urinary albumin/creatinine ratio had a higher predictive value ($\beta$=-0.84, $p<0.001$) than change in diastolic blood pressure ($\beta$=-0.19, p=0.018). In addition baseline HbA1c ($\beta$=-0.178, p=0.023) and baseline systolic blood pressure ($\beta$=-0.184, p=0.029) also influenced improvement in urinary albumin/creatinine ratio.

From the cohort of 43 patients who were treated with liraglutide for 6 months, 35 patients continued treatment for at least 12 months. This group of 35 patients were matched with suitable controls at baseline and the above mentioned parameters were analysed at 12 month follow up.

After 12 months the changes in urinary albumin/creatinine ratios did not differ between the liraglutide treated group and the control group. After 12 months the liraglutide treatment group showed a significant reduction in HbA1c of 4.44 mmol/mol, but in the control group an increase in HbA1c of 4.00 mmol/mol was noted. The reduction in BMI noted at 6 months in the liraglutide group was maintained- the reduction in BMI at 12 months was 1.04 $kg/m^2$. There were no statistically significant changes in total cholesterol, systolic blood pressure and diastolic blood pressure between the two groups. The results are displayed on table 3, figures 3 and 4.

| DOMAIN | LIRAGLUTIDE Mean difference (Standard error) | CONTROL Mean difference (Standard error) | Significance p= |
|---|---|---|---|
| **Urinary Albumin/Creatinine (mg/mmol)** | -5.04 (1.88) | -7.17 (2.73) | 0.52 (NS) |
| **BMI (kg/m2)** | -1.04 ± 0.36 | +0.08 ± 0.27 | 0.01 |
| **HbA1c (mmol/mol)** | -4.44 ± 2.68 | +4.00 ± 2.59 | 0.02 |
| **Systolic Blood Pressure (mm Hg)** | -5.14 ± 2.23 | -1.20 ± 2.80 | 0.20 (NS) |
| **Diastolic Blood Pressure (mm Hg)** | -2.00 ± 1.91 | +0.52 ± 2.39 | 0.41 (NS) |
| **Total Cholesterol (mmol/L)** | -0.09 ± 0.11 | -0.09 ± 0.15 | 0.99 (NS) |

Table 3.3: CHANGES IN PRIMARY AND SECONDARY ENDPOINTS FROM BASELINE TO 12 MONTH FOLLOW UP. Statistically significant improvements in HbA1c and BMI were noted in the liraglutide treated patients at 12 months, when compared with controls.

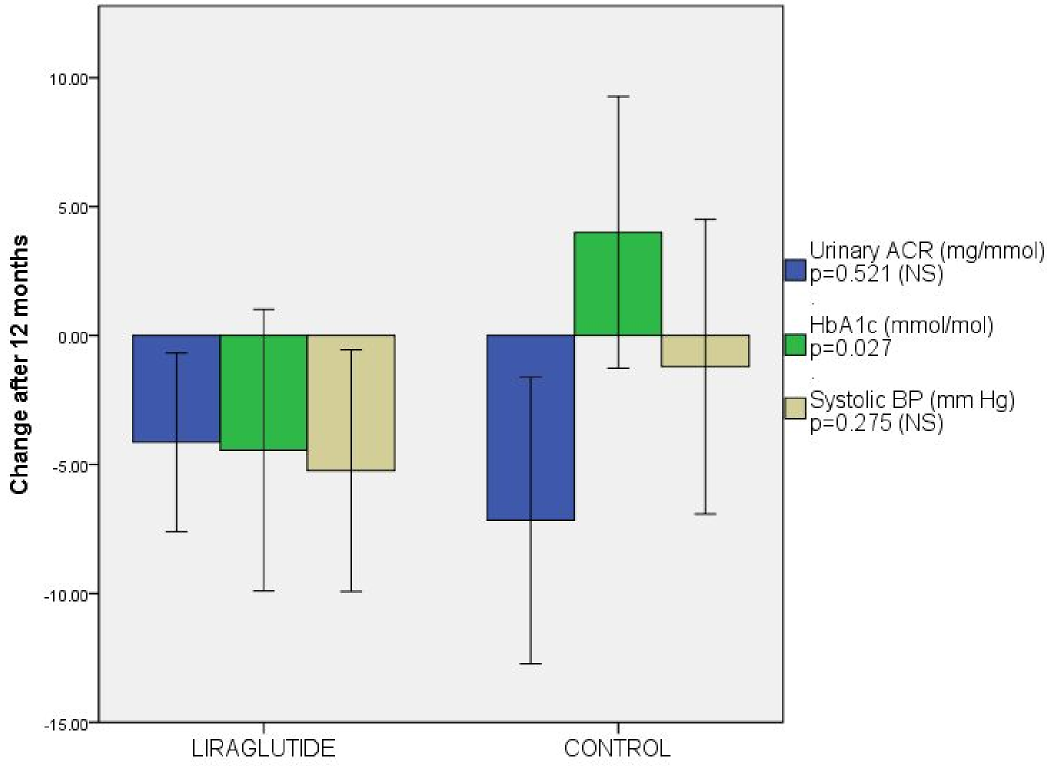



Figure 3.3: CHANGES IN ACR, HBA1C AND SYSTOLIC BP AFTER 12 MONTHS. The primary endpoint-urinary albumin/creatinine ratio was reduced in both liraglutide group and control group, however there was no difference between groups. No significant differences in systolic blood pressures were noted.

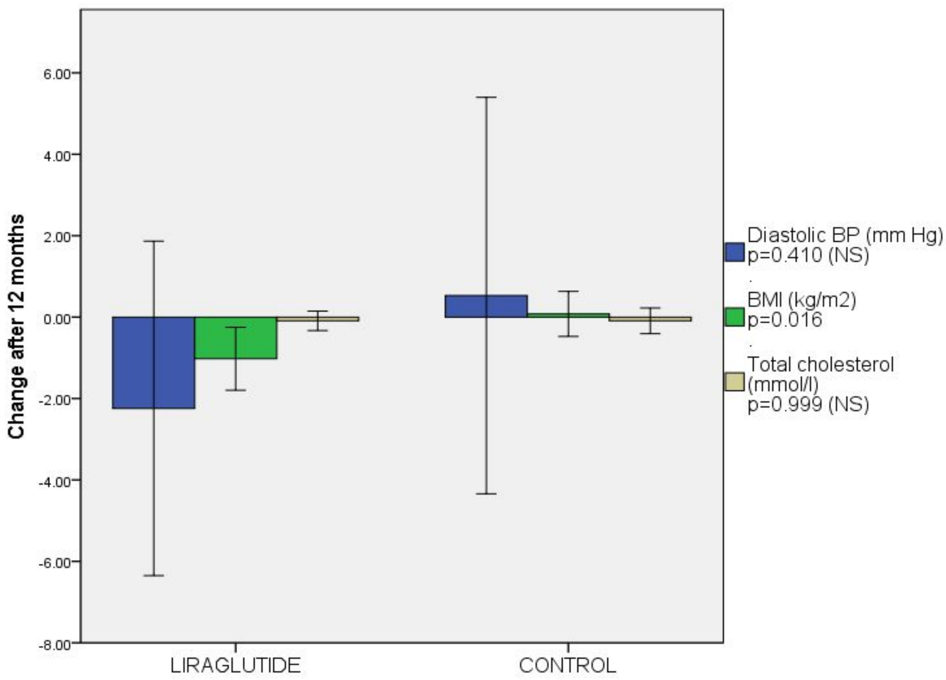



Figure 3.4: CHANGES IN DIASTOLIC BP, BMI AND TOTAL CHOLESTEROL AFTER 12 MONTHS. The significant improvement noted at 6 months was maintained at 12 months. However no changes in diastolic blood pressures and cholesterol levels were noted between groups.

I prospectively investigated whether treatment with liraglutide modulates the markers of endothelial dysfunction and inflammation, thereby influencing the progression of nephropathy over the next few years. The above mentioned biomarkers in serum were measured in 11 subjects (6 males and 5 females) with established microalbuminuria at baseline and 3 months after treatment with liraglutide. The results are shown on table 4 and figure 5.

| DOMAIN n=11 | BASELINE Mean (Standard Deviation) | 3 MONTHS AFTER LIRAGLUTIDE Mean (Standard Deviation) | CHANGE AFTER 3 MONTHS Mean (95% Confidence intervals) | Significance. p= |
|---|---|---|---|---|
| **BMI (kg/m2)** | 48.78 ± 17.55 | 46.86 ± 15.67 | -1.91 (-3.56 to -0.27) | 0.02 |
| **HbA1c mmol/mol** | 74.18 ± 24.22 | 58.09 ± 12.66 | -16.09 (-28.18 to -3.99) | 0.01 |
| **Systolic Blood Pressure (mmHg)** | 153.60 ± 17.98 | 141.20 ± 16.00 | -12.40 (-26.20 to +1.40) | 0.07 (NS) |
| **Diastolic Blood Pressure (mmHg)** | 79.00 ± 10.57 | 71.40 ± 11.98 | -7.60 (-16.20 to +1.00) | 0.08 (NS) |
| **Total Cholesterol (mmol/l)** | 4.00 ± 0.76 | 3.60 ± 0.84 | -0.40 (-0.83 to +0.03) | 0.07 (NS) |
| **LDL Cholesterol (mmol/l)** | 1.97 ± 0.50 | 1.73 ± 0.62 | -0.23 (-0.58 to +0.11) | 0.16 (NS) |
| **Urinary Albumin/Creatinine (mg/mmol)** | 6.15 ± 4.31 | 3.40 ± 4.03 | -2.74 (-5.62 to +0.12) | 0.06 (NS) |
| **sICAM-1 (ng/ml)** | 949.25 ± 366.86 | 1018.34 ± 281.06 | +69.09 (-125.24 to +263.43) | 0.44 (NS) |
| **sVCAM-1 (ng/ml)** | 832.79 ± 304.48 | 950.61 ± 309.25 | +117.81 (-17.82 to +253.45) | 0.08 (NS) |
| **MCP-1 (pg/ml)** | 90.05 ± 30.42 | 104.02 ± 41.43 | +13.97 (-4.58 to +32.53) | 0.12 (NS) |
| **hsCRP (mg/l)** | 5.50 ± 4.27 | 5.12 ± 5.07 | -0.37 (-4.36 to +5.11) | 0.85 (NS) |

Table 3.4: CLINICAL PARAMETERS AND SERUM BIOMARKERS IN SUBJECTS WITH ABNORMAL URINARY ALBUMIN/CREATININE AT BASELINE AND 3 MONTHS AFTER TREATMENT. There were improvements in BMI and HbA1c level of liraglutide treated subjects. However other clinical parameters were unchanged.

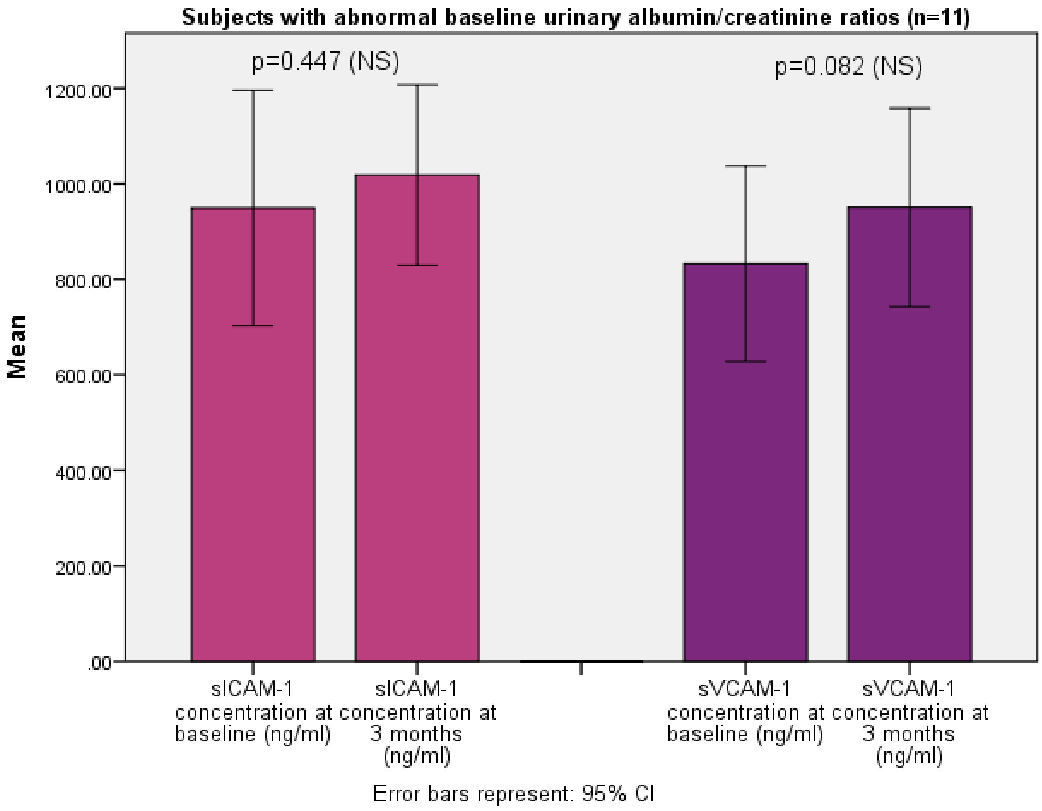



Figure 3.5: BASELINE AND POST TREATMENT LEVELS OF BIOMARKERS OF ENDOTHELIAL DYSFUNCTION. No significant differences were noted in circulating levels of sICAM-1 and sVCAM-1 3 months post-treatment with liraglutide.

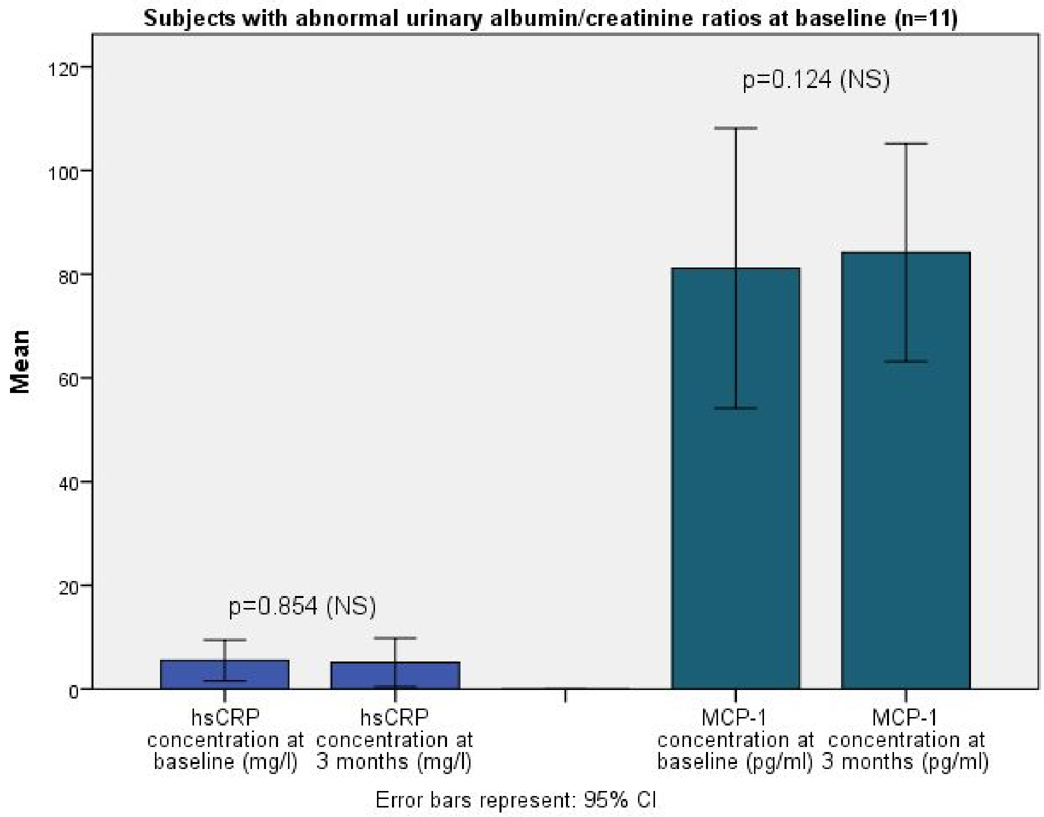



Figure 3.6: BASELINE AND POST TREATMENT LEVELS OF BIOMARKERS OF INFLAMMATION. No significant changes in circulating levels of hsCRP or MCP-1 were noted 3 months post-treatment with liraglutide.

When compared with baseline values, there was no significant change in plasma levels of sICAM-1, sVCAM-1, MCP-1 or hsCRP after treatment (results shown on figures 5 and 6). There was a statistically significant reduction in BMI and improvement in HbA1c at 3 months (results shown on table 4). There was a trend towards improvement in systolic and diastolic blood pressures after treatment (results shown on table 4).

**Discussion**

This is the first human study to investigate the effect of GLP-1 agonists on the progression of urinary ACR. Although previous animal studies suggested a possible protective effect of GLP-1 based treatments on progression of nephropathy, our retrospective cohort study did not show any benefit.

**Predictors of improvement in urinary ACR**

Regression analysis of the data indicated that the total model was robust explaining 70% of the primary outcome variable which was change in urinary albumin/creatinine ratio. After controlling for the effects of age, duration of diabetes, baseline blood pressures and baseline HbA1c, the most reliable predictor of change in albumin excretion in our cohort was the baseline urinary ACR. This is in keeping with a Japanese retrospective cohort study that identified baseline urinary albumin/creatinine ratio as the most significant predictor of progression of diabetic nephropathy (Forsblom, Groop et al. 1998, Yamada, Komatsu et al. 2005). This indicates that patients with persistent microalbuminuria were identified by the clinician and given targeted treatment in an effort to improve the renal outcome.

A Japanese study also identified baseline HbA1c and average systolic blood pressure over 8 years as important predictors of regression in urinary albumin excretion (Yamada, Komatsu et al. 2005). In our cohort we have confirmed that baseline HbA1c is a key parameter in altering the course of diabetic nephropathy. In addition we identified that, change in diastolic blood pressure is an independent predictor of change in urinary albumin excretion. It is well known that tight blood pressure control in patients with hypertension and type 2 diabetes achieves a clinically important reduction in the progression of microalbuminuria(1998, Cooper 1998, Gray, Raikou et al. 2000). Drugs acting on the renin-angiotensin axis are currently the first line treatment for patients with diabetes and microalbuminuria and might possible confer benefits over and above simple blood pressure reduction (Brenner, Cooper et al. 2001, Chan, Wat et al. 2004, Jerums, Allen et al. 2004, Casas, Chua et al. 2005). In our study population all patients were treated with either an angiotensin convertase inhibitor or angiotensin receptor blocking drug as per guidelines.

Diabetic nephropathy is a chronic complication that gradually progress over years or decades. It is estimated that about 80% of patients with established microalbuminuria have an increase in their urinary albumin excretion by 10-20% every year. This happens over a period of 10-15 years culminating in clinical albuminuria and is associated with a gradual rise in blood pressure (Molitch, DeFronzo et al. 2004). Urinary albumin is the most widely used marker of diabetic nephropathy in clinical practice and correlates well with progression of diabetes related renal injury (Bakker 1999). In our cohort of patients with established microalbuminuria, treatment with liraglutide for up to

12 months did not make any significant change in urinary albumin/creatinine ratio. In view of the long natural history of the disease it is possible that one year is too short a period to detect a significant change. A longitudinal study lasting several years is required to conclusively demonstrate whether liraglutide has an effect on urinary albumin excretion rate. However a trend towards the rate of progression of nephropathy might be indicated by certain biomarkers.

In this study a significant improvement in the mean HbA1c was noted in the liraglutide treatment group when compared with the control group. This is possibly due to the increased frequency of healthcare contacts in the liraglutide treatment group. Several previous studies have noted improvements in diabetes control that were directly attributed to greater input from healthcare professionals (Kaufman, Halvorson et al. 1999, Wagner, Grothaus et al. 2001).

**Markers of inflammation and endothelial dysfunction**

It has been shown that endothelial dysfunction and inflammation predate the onset and progression of diabetic nephropathy. A rise in serum levels of sICAM-1 and sVCAM-1 which are bio-markers of endothelial dysfunction has been noted several years before an increase urinary albumin excretion was detected (Astrup, Tarnow et al. 2008, Persson, Rossing et al. 2008). Conversely, lower levels of these markers are associated with slower progression of nephropathy (Chow, Nikolic-Paterson et al. 2005). Several cross-sectional studies in humans have shown a significant correlation between biomarkers of inflammation such as hsCRP and MCP-1 and severity of urinary albumin excretion (Chiarelli, Cipollone et al. 2002, Kiyici, Erturk et al. 2006, Goldberg 2009, Kajitani, Shikata et al. 2010). Blood level of hsCRP is a well-known marker of micro inflammation and is closely related to severity of diabetic nephropathy (Kajitani, Shikata et al. 2010). The irbesartan in patients with type 2 diabetes and microalbuminuria study was a 2 year prospective, randomized controlled trial that compared irbesartan with placebo. The primary end point was time to onset of diabetic nephropathy. A post-hoc, analysis of data from the study suggested that hsCRP might predict progression of microalbuminuria (Persson, Rossing et al. 2008). Some of these biomarkers have been studied in animal models.

Diabetic rats treated with exendin-4 for 8 weeks showed a significant improvement in the urinary albumin excretion rate when compared with controls. This improvement was noted even though there was no significant change in fasting glucose or glycated haemoglobin. Renal histology demonstrated that glomerular hypertrophy, mesangial matrix expansion and inflammatory cell infiltration in the kidney were lower in the exendin-4 treated rats (Park, Kim et al. 2007). In a rat model of diabetic nephropathy, the injection of GLP-1 agonist exendin-4 exerted renoprotective effects through an anti-inflammatory action. Exendin-4 acted directly on the GLP-1 receptor in the rat kidney and attenuated the production of pro inflammatory cytokines and ICAM-1(Kodera, Shikata et al. 2011). Rats with diabetes treated with subcutaneous injections of liraglutide for 4 weeks showed a reduction in oxidative stress markers, transforming growth factor-β and urinary albumin excretion (Hendarto, Inoguchi et al. 2012).

Sitagliptin is a selective inhibitor of the enzyme dipietidyl pepdidase-4 which inactivates GLP-1. Spontaneously hypertensive rats treated with sitagliptin showed an improvement in endothelium dependent relaxation of renal arteries and restored renal blood flow. Ex vivo exendin-4 treatment also improved endothelial function of renal arteries from human subjects with hypertension (Liu, Liu et al. 2012).

Thus there is a significant body of evidence from animal models and ex vivo studies suggesting that GLP-1 might modulate the course of diabetic nephropathy. We hypothesised that liraglutide treatment in human subjects with type 2 diabetes might have an impact on the biomarkers of endothelial dysfunction (ICAM-1 and VCAM-1) and inflammation (MCP-1 and hsCRP), thereby altering the progression of microalbuminuria. But in our prospective study involving 11 human subjects with type 2 diabetes and established microalbuminuria, there was no change in the serum levels of the above mentioned biomarkers before and 3 months after treatment with liraglutide.

**Conclusion**

In patients with type 2 diabetes and established microalbuminuria, treatment with liraglutide does not influence the progression of urinary albumin excretion. It is known that glomerular hyperfiltration and hyperperfusion associated with subtle morphological changes in the glomerular basement membrane and mesangium occurs many years prior to appearance of abnormal urinary albumin excretion(Cooper 1998). It is estimated that subjects who are normoalbuminuric at the time of diagnosis of diabetes remain free of overt nephropathy for about 19 years(Adler, Stevens et al. 2003). To further evaluate the influence of liraglutide in diabetic nephropathy we studied its effect in a group of subjects with normal urinary albumin excretion. This is discussed in the next chapter.

# CHAPTER 4

## EFFECT OF LIRAGLUTIDE ON DEVELOPMENT OF MICROALBUMINURIA IN PATIENTS WITH TYPE 2 DIABETES AND NORMAL URINARY ALBUMIN EXCRETION RATES

## Introduction

Diabetes related renal injury goes through several distinct phases of development. Functional changes in the nephron in the form of hyperperfusion and glomerular hyperfiltration occur before there are any detectable structural or clinical changes (Mauer, Steffes et al. 1984). Hyperfiltration is due to a decrease in resistance offered by the afferent and the efferent arterioles, the reduction being greater in the efferent arteriole (Ziyadeh and Wolf 2008). Many factors are involved in this defective auto regulation including angiotensin 2, cytokines and pro inflammatory molecules (Wolf, Kalluri et al. 1999, Wolf and Ziyadeh 1999, Ziyadeh and Wolf 2008). Hyperfiltration leads to appearance of albumin in the urine, which over a period of time gradually increases to become pathological.

Work on animal models suggests that GLP-1 analogues may prevent disease progression in the early stages of albuminuria (Kodera, Shikata et al. 2011). This is thought to be due to modulation of inflammation and endothelial dysfunction. In this study I explored the effect of liraglutide on early diabetes related renal injury in subjects with type 2 diabetes.

## Aim

In this study I explored the effect of the GLP-1 analogue liraglutide on progression of urinary ACR in patients with detectable urinary albumin excretion, but not diagnostic of pathological microalbuminuria.

**Methods**

This study was similar to the study on the effect of liraglutide on progression of microalbuminuria in patients with type 2 diabetes. I included patients with type 2 diabetes and normoalbuminuria who were treated with liraglutide for at least 6 months. Cases selection for the retrospective study is shown in the flowchart below.

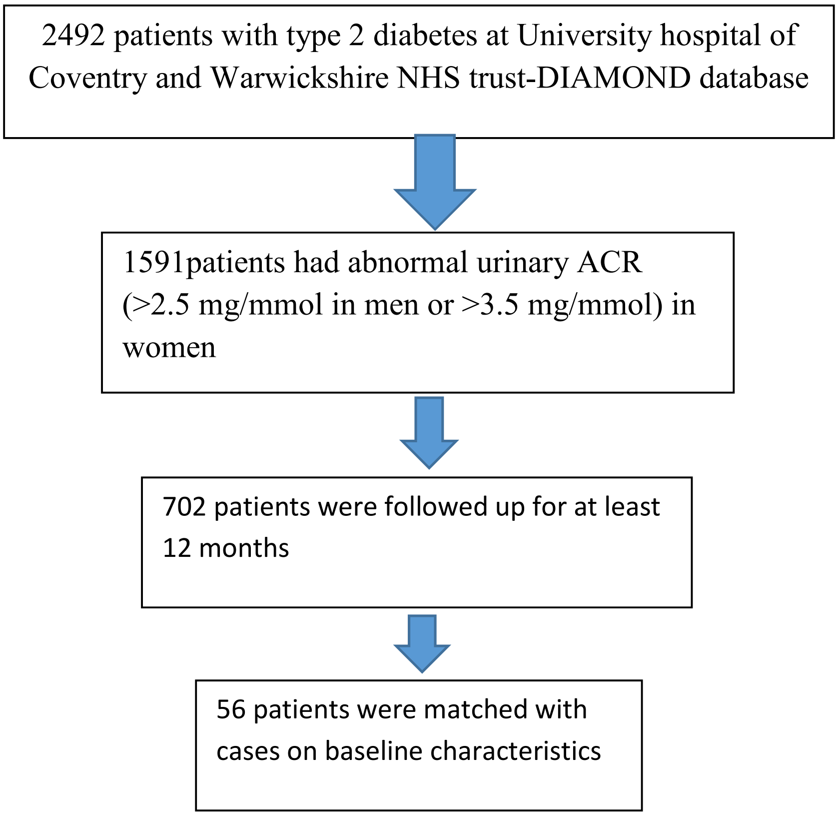
402 patients treated with liraglutide at University hospital of Coventry and Warwickshire NHS trust

233 patients had normal urinary ACR (<2.5 mg/mmol in men or <3.5 mg/mmol) in women

131 patients were followed up for at least 6 months

63 patients had study outcome dataset recorded at baseline, 6 and 12 months

They were compared with a matched group of subjects who were not treated with liraglutide. Controls were selected from the database of patients with type 2 diabetes on the DIAMOND database at University hospital of Coventry and Warwickshire. Selection of controls is shown in the flowchart below.




Primary outcome

2. Changes in urinary albumin creatinine ratios after 6 and 12 months.

Secondary outcome

2. Changes in BMI, HbA1c, total cholesterol, systolic and diastolic blood pressures after 6 and 12 months

All patients attending the GLP-1 clinic to commence treatment with liraglutide were invited to participate in the prospective study. Details of patient recruitment are detailed in chapter 2-Methods. 25 patients had normoalbuminuria, out of which, 9 patients agreed to participate after informed consent. The biomarkers of diabetes related kidney injury were measured before and 3 months after treatment with liraglutide.

Each serum sample was assayed for the molecule of interest in duplicate and the mean of the two values was used for statistical analysis. If the duplicates varied by >10%, that particular sample was excluded from the analysis.

Primary outcomes

3. Changes in biomarkers of endothelial dysfunction (sICAM-1 an sVCAM-1) after 3 months of treatment with liraglutide.
4. Changes in biomarkers of inflammation (hsCRP and MCP-1) after 3 months of treatment with liraglutide.

Secondary outcomes

3. Changes in urinary albumin creatinine ratios after 3 months of treatment with liraglutide
4. Changes in BMI, HbA1c, total cholesterol, systolic and diastolic blood pressures after 3 months of treatment with liraglutide.

**Results**

In a retrospective cohort study, baseline and 6 month follow up data was collected for a group of 63 patients (30 males, 33 females) with normoalbuminuria, who were treated with liraglutide. They were matched with 56 patients (29 males, 27 females) with type 2 diabetes who were not treated with liraglutide. All cases and controls were treated with either an ACE-I or ARB for hypertension. Baseline characteristics of the study groups are shown on table 1.

| DOMAIN | LIRAGLUTIDE Mean (Standard Deviation) | CONTROL Mean (Standard Deviation) |
|---|---|---|
| **Number** | 63 | 56 |
| **Males:Females (number)** | 30:33 | 29:27 |
| **Age (years)** | 58.92 ± 8.55 | 61.96 ± 9.23 |
| **Duration of Diabetes (years)** | 12.43 ± 8.29 | 12.13 ± 8.02 |
| **Weight (kg)** | 107.73 ± 21.33 | 101.49 ± 17.60 |
| **BMI (kg/m2)** | 38.54 ± 5.96 | 36.61 ± 4.60 |
| **HbA1c (mmol/mol)** | 70.43 ± 17.49 | 64.66 ± 14.28 |
| **Systolic Blood Pressure (mm Hg)** | 144.03 ± 18.81 | 137.98 ± 15.02 |
| **Diastolic Blood Pressure (mm Hg)** | 76.68 ± 10.56 | 77.07 ± 12.26 |
| **Total Cholesterol (mmol/L)** | 4.26 ± 0.99 | 4.19 ± 0.97 |
| **Urinary Albumin/Creatinine (mg/mmol)** | 0.81 ± 0.49 | 0.97 ± 0.57 |

Table 4.1: BASELINE CHARACTERISTICS OF CASES AND CONTROLS WITH NORMAL ALBUMIN:CREATININE RATIO

Normality of distribution of the baseline variables were assessed by Kolmogorov-Smirov and Shapiro-Wilk tests using SPSS software (threshold for significance was 0.05). If the 'p' values for both tests were non-significant, these variables were considered normally distributed for the purpose of this study.

After 6 months there was a statistically significant reduction the urinary albumin excretion rates of subjects in both groups. The mean baseline ACR for all participants (liraglutide treated group and control group) was 0.88 mg/mmol, whereas the mean ACR after 6 months was 1.02 mg/mmol. The mean change in ACR was 0.138 mg/mmol (95% CI: -0.21 to -0.01, $p=0.048$).

At 6 months, the mean albumin/creatinine ratio in the liraglutide group was reduced by 0.03 mg/mmol, whereas it increased by 0.29 mg/mmol in the control group. This showed a trend towards statistical significance with a p value of 0.06. Change in BMI was significantly different with a decrease in BMI of 1.28 kg/m$^2$ in the liraglutide group and an increase in BMI of 0.15 kg/m$^2$ in the control group. The patients in liraglutide group had a reduction in mean HbA1c of 7.53 mmol/mol, whereas in the control group mean HbA1c increased by 3.00 mmol/mol. No statistically significant changes in systolic blood pressure, diastolic blood pressure or change in total cholesterol were noted between the two groups. The results are shown on table 2, figures 1 and 2.

| DOMAIN | LIRAGLUTIDE Mean difference (Standard error) | CONTROL Mean difference (Standard error) | Significance p= |
|---|---|---|---|
| **Urinary Albumin/Creatinine (mg/mmol)** | -0.03 ± 0.06 | +0.29 ± 0.15 | 0.072 |
| **BMI (kg/m2)** | -1.28 ± 0.24 | +0.15 ± 0.13 | 0.00 |
| **HbA1c (mmol/mol)** | -7.53 ± 1.44 | +3.00 ± 1.49 | 0.00 |
| **Systolic Blood Pressure (mm Hg)** | -3.57 ± 1.98 | -0.07 ± 1.52 | 0.17 |
| **Diastolic Blood Pressure (mm Hg)** | -1.17 ± 1.28 | +0.57 ± 1.15 | 0.31 |
| **Total Cholesterol (mmol/L)** | -0.31 ± 0.11 | -0.18 ± 0.08 | 0.36 |

Table 4.2: CHANGES IN PRIMARY AND SECONDARY ENDPOINTS FROM BASELINE TO 6 MONTH FOLLOW UP. There were significant improvements in the primary endpoint-change in urinary albumin/creatinine ratios in both the liraglutide group and control group. However no significant chnages were noted between the two groups.

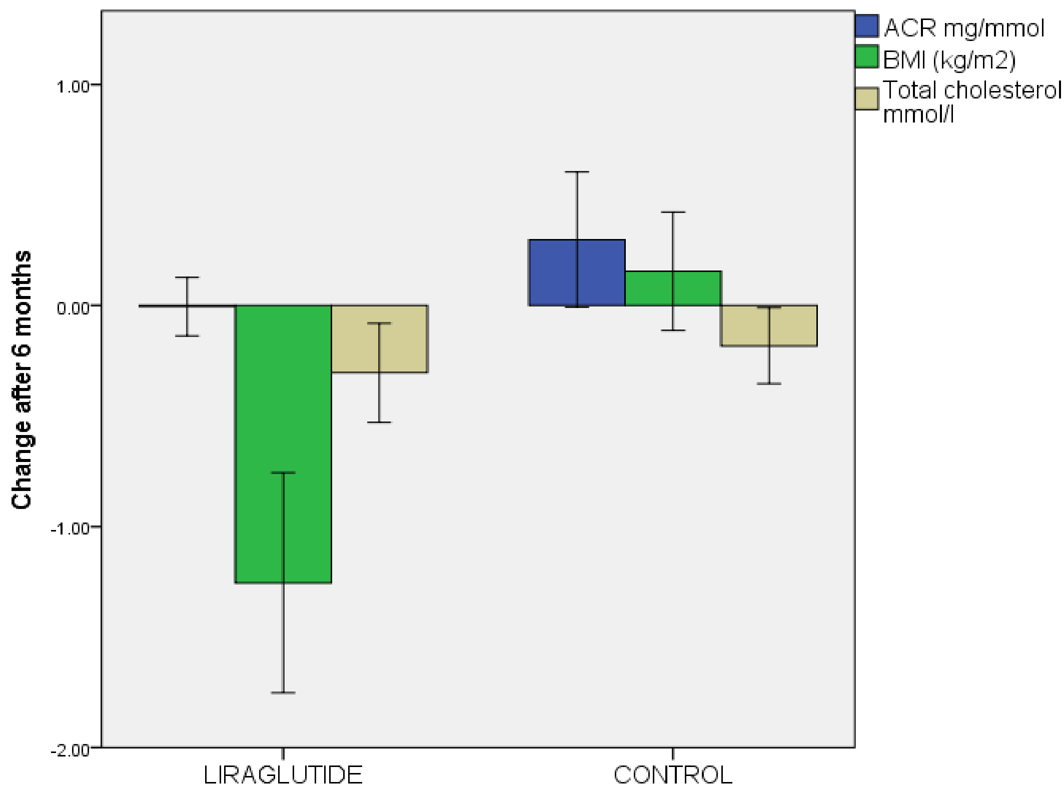



Figure 4.1: CHANGES IN ACR, BMI AND TOTAL CHOLESTEROL AFTER 6 MONTHS. After 6 months, significant improvements in BMI were noted in the liraglutide treatment group, however no changes were evident in total cholesterol or urinary albumin/creatinine ratios.

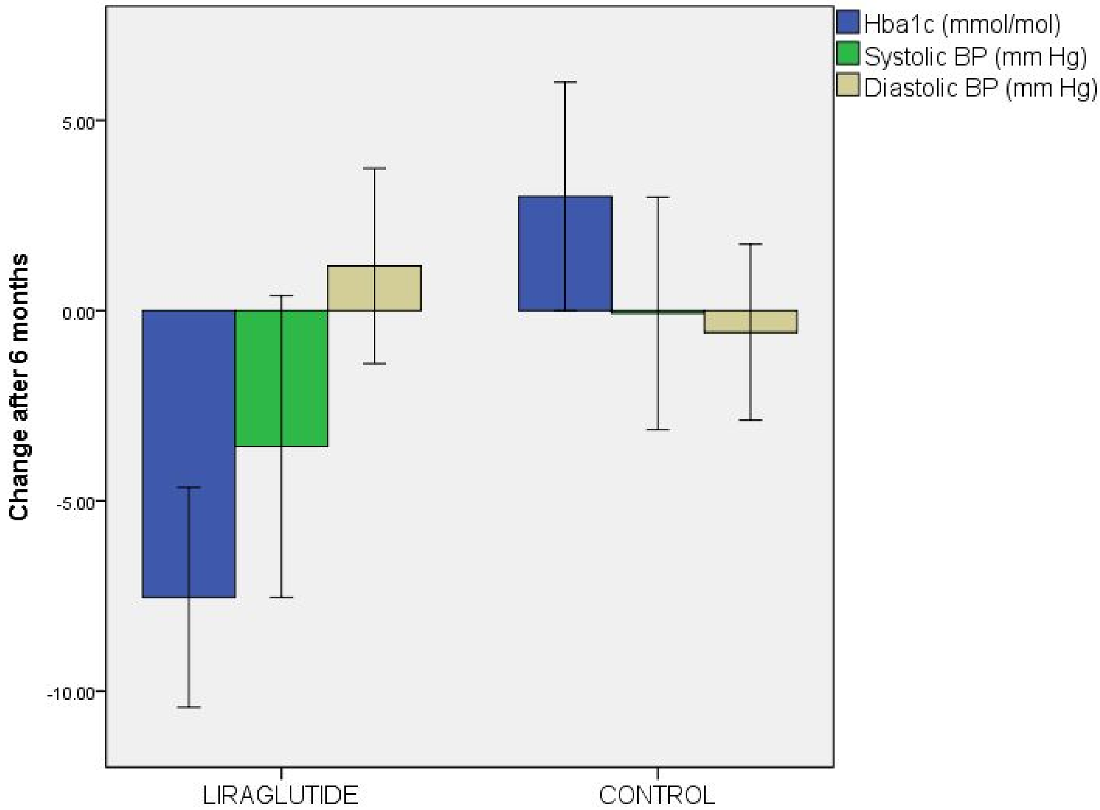



Figure 4.2: CHANGES IN HBA1C, SYATOLIC AND DIASTOLIC BLOOD PRESSURES AFTER 6 MONTHS. There was a significant improvement in HbA1c in the liraglutide treated group when compared with controls. However no changes in systolic or diastolic blood pressures were evident.

Out of the 63 patients treated with liraglutide for 6 months, 57 continued treatment for at least 12 months. Baseline characteristics of this group of 57 patients were matched with a group of 57 controls and the above noted parameters were analysed at 12 months. After 12 months the changes in albumin/creatinine ratios was not statistically different between the liraglutide treatment group and the control group. The liraglutide treated patients maintained the statistically significant reductions in BMI and HbA1c that were noted at 6 months. No statistically significant changes in systolic blood pressure, diastolic blood pressure or total cholesterol were evident between the two groups. The results are shown on table 3, figures 3 and 4.

| DOMAIN | LIRAGLUTIDE Mean difference (Standard error) | CONTROL Mean difference (Standard error) | Significance p= |
|---|---|---|---|
| **Urinary Albumin/Creatinine (mg/mmol)** | -0.07 ± 0.10 | -0.35 ± 0.13 | 0.11 |
| **BMI (kg/m2)** | -1.26 ± 0.26 | +0.27 ± 0.17 | 0.00 |
| **HbA1c (mmol/mol)** | -5.09 ± 1.56 | +3.28 ± 1.51 | 0.00 |
| **Systolic Blood Pressure (mm Hg)** | -2.80 ± 1.65 | -1.54 ± 1.93 | 0.62 |
| **Diastolic Blood Pressure (mm Hg)** | -1.75 ± 1.37 | -2.80 ± 1.44 | 0.59 |
| **Total Cholesterol (mmol/L)** | -0.04 ± 0.12 | -0.12 ± 0.07 | 0.60 |

Table 4.3: CHANGES IN PRIMARY AND SECONDARY ENDPOINTS FROM BASELINE TO 12 MONTH FOLLOW UP. Statistically significant reductions in urinary albumin/creatinine ratios were evident in both groups, but no differences were noted between groups.

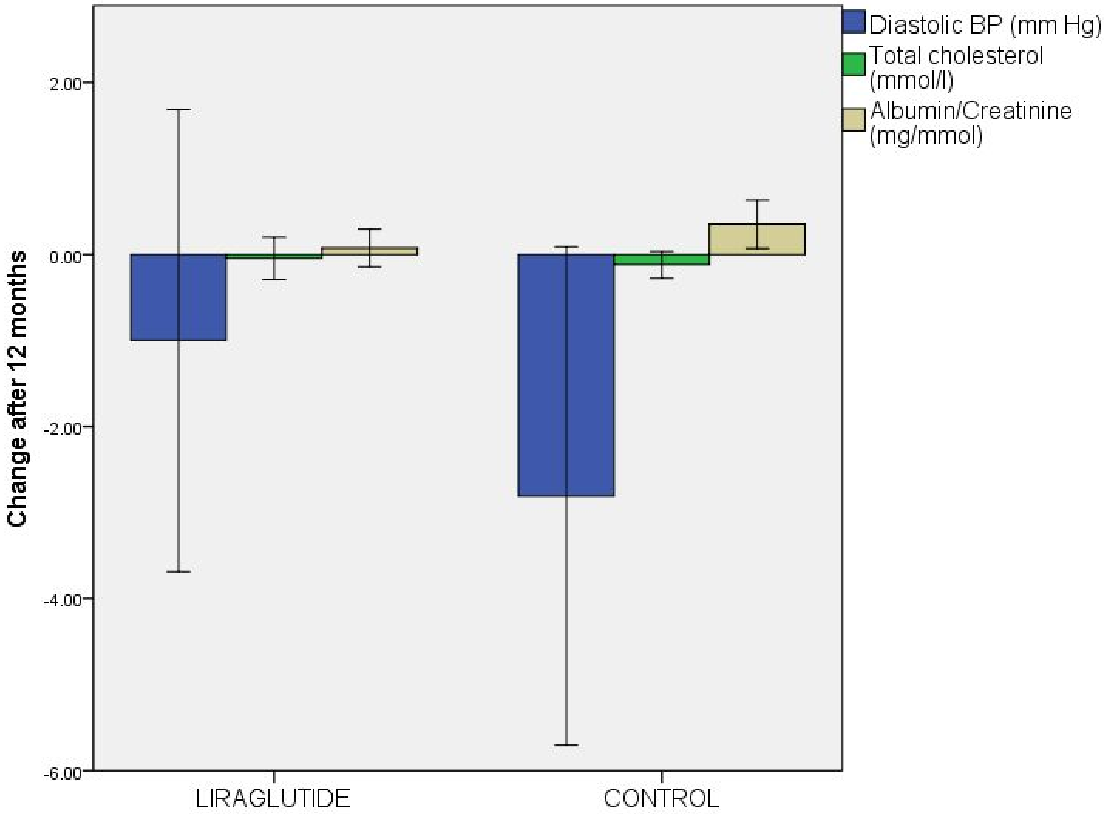



Figure 4.3: CHANGES IN ACR, DIASTOLIC BLOOD PRESSURE AND TOTAL CHOLESTEROL AFTER 12 MONTHS. Changes in total cholesterol and diastolic blood pressures were not different between groups.

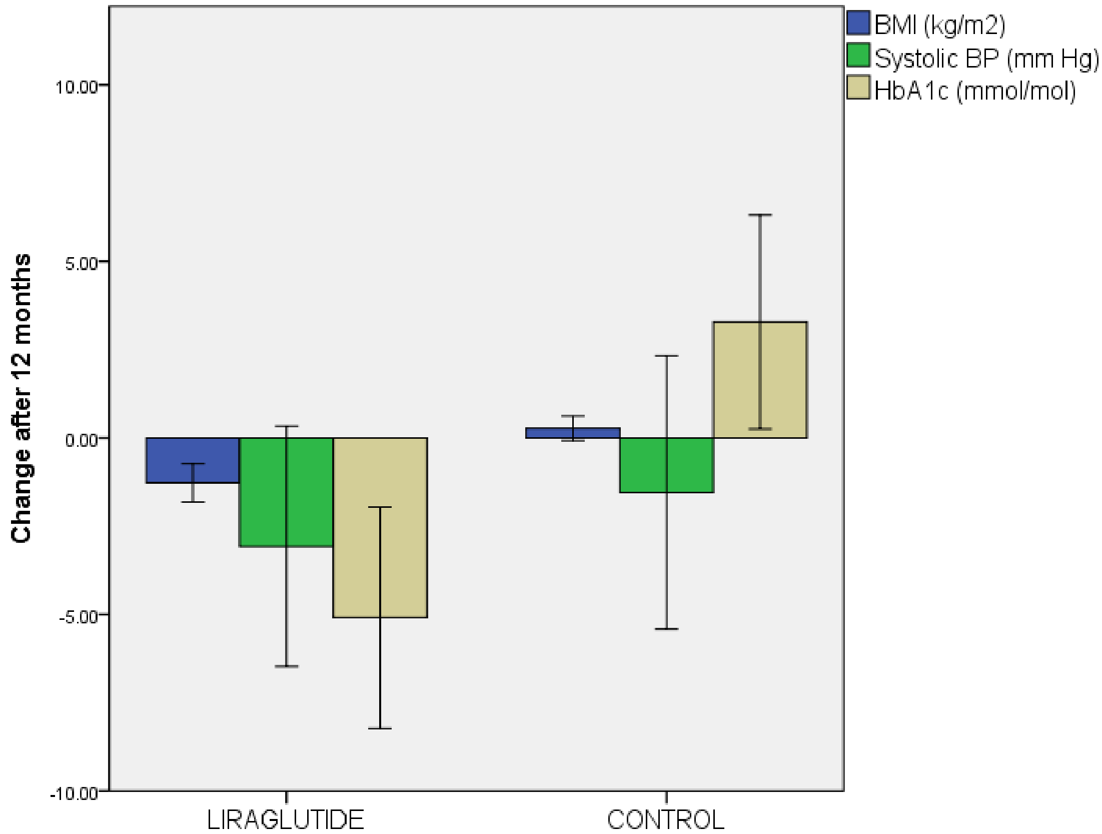



Figure 4.4: CHANGES IN BMI, SYSTOLIC BLOOD PRESSURE AND HBA1C AFTER 12 MONTHS. Significant improvements in mean BMI and HbA1c were noted in the liraglutide treated patients when compared with controls. No differences in systolic blood pressures were evident between groups.

Hierarchical logistic regression was performed to assess whether liraglutide treated subjects had a significant change in urinary albumin excretion. The specified dependent variable was improvement or worsening of urinary ACR after 12 months. Age, duration of diabetes, baseline HbA1c, baseline urinary albumin/creatinine ratio, baseline systolic and diastolic blood pressures were entered as independent variables at step1. After controlling for these variables, change in HbA1c, changes in systolic and diastolic blood pressures were entered as independent variables in step 2. After step 2 the total variance explained by the model as a whole was 17%, $F$ (9, 96) =2.10, p=0.036. After controlling for all the above mentioned variables, treatment with liraglutide was entered as the independent variable at step 3. This explained an additional 2% variance in urinary albumin/creatinine ratio only, $F$ (10, 95) =2.21, p=0.023. In the final model baseline urinary albumin/creatinine ratio and change in diastolic blood pressure were highly statistically significant.
Baseline urinary albumin/creatinine ratio had a higher predictive value ($\beta$=-0.283, p=0.004) than change in diastolic blood pressure ($\beta$=0.264, p=0.024). In addition the predictive value of baseline HbA1c ($\beta$=0.188, p=0.07) approached statistical significance.

The biomarkers of endothelial dysfunction and inflammation in were measured at baseline and 3 months after liraglutide therapy 9 subjects (4 males and 5 females) with type 2 diabetes and normal albumin/creatinine ratio. The results are shown on table 4 and figure 5.

| DOMAIN<br>n=11 | BASELINE<br>Mean (Standard Deviation) | 3 MONTHS AFTER LIRAGLUTIDE<br>Mean (Standard Deviation) | CHANGE AFTER 3 MONTHS<br>Mean (95% Confidence intervals) | Significance. p= |
|---|---|---|---|---|
| **BMI (kg/m2)** | 41.83 ± 7.03 | 40.19 ± 7.11 | -0.92 (-1.63 to -0.21) | 0.01 |
| **HbA1c mmol/mol** | 69.70 ± 10.94 | 58.30 ± 11.32 | -11.4 (-18.07 to -4.73) | 0.00 |
| **Systolic Blood Pressure (mmHg)** | 145.44 ± 21.01 | 137.89 ± 19.97 | -7.55 (-20.99 to +5.88) | 0.23 |
| **Diastolic Blood Pressure (mmHg)** | 75.33 ± 10.94 | 73.78 ± 15.25 | -1.55 (-8.36 to +5.25) | 0.61 |
| **Total Cholesterol (mmol/l)** | 4.45 ± 1.25 | 4.29 ± 1.26 | -0.16 (-0.83 to +0.51) | 0.6 |
| **LDL Cholesterol (mmol/l)** | 2.21 ± 0.79 | 2.27 ± 1.05 | +0.06 (-0.50 to +0.62) | 0.81 |
| **Urinary Albumin/Creatinine (mg/mmol)** | 0.98 ± 0.52 | 0.90 ± 0.74 | -0.07 (-0.79 to +0.63) | 0.81 |
| **sICAM-1 (ng/ml)** | 1306.38 ± 729.84 | 1481.25 ± 572.68 | +174.86 (-111.18 to +460.91) | 0.19 |
| **sVCAM-1 (ng/ml)** | 947.66 ± 414.53 | 1301.86 ± 627.43 | +354.19 (+79.28 to +629.21) | 0.02 |
| **MCP-1 (pg/ml)** | 117.54 ± 39.28 | 84.03 ± 34.03 | -33.50 (-77.76 to +10.76) | 0.11 |
| **hsCRP (mg/l)** | 6.04 ± 3.51 | 4.86 ± 3.54 | -1.17 (-0.48 to+2.83) | 0.14 |

Table 4.4: CLINICAL PARAMETERS AND SERUM BIOMARKERS IN SUBJECTS WITH NORMAL URINARY ALBUMIN/CREATININE AT BASELINE AND 3 MONTHS AFTER TREATMENT. sICAM-1 levels remained unchanged after 3 months of treatment. A statistically significant increase in sVCAM-1 was noted after treatment. No changes were evident in circulating levels of biomarkers of inflammation.

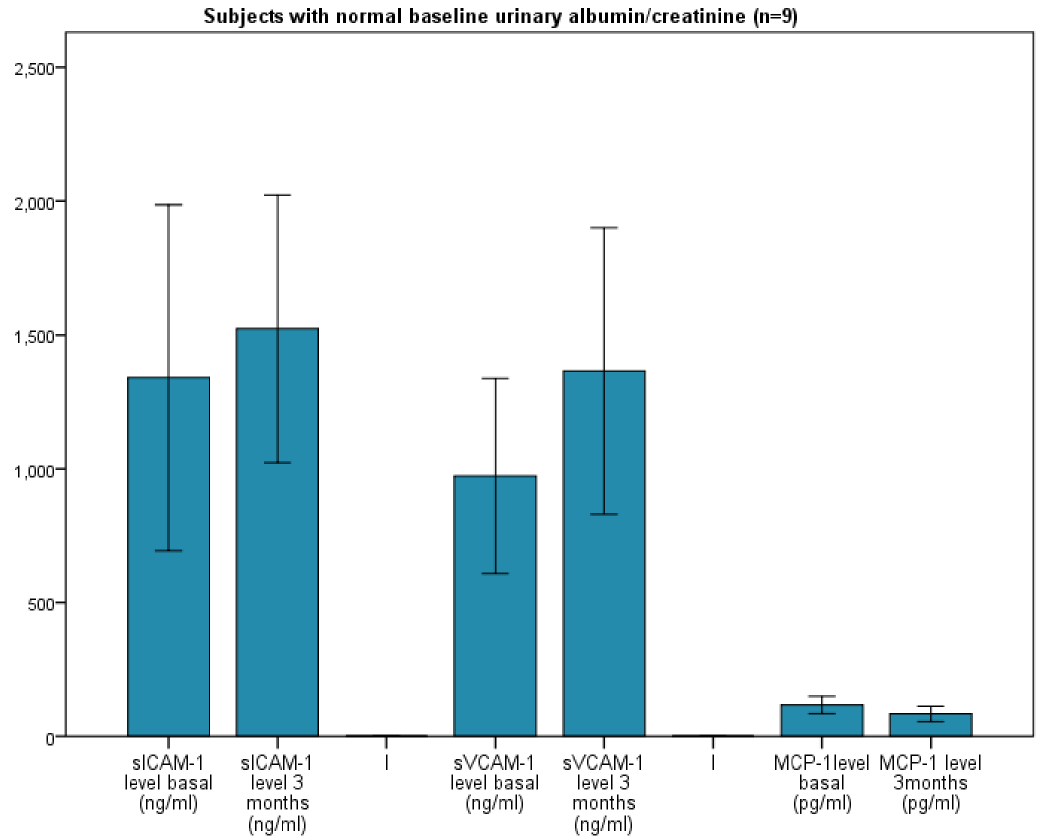



Figure 4.5: BASELINE AND POST TREATMENT LEVELS OF BIOMARKERS OF ENDOTHELIAL DYSFUNCTION AND INFLAMMATION. No significant changes in circulating levels of the above mentioned biomarkers were evident 3 months after treatment with liraglutide.

There was a statistically significant increase in plasma sVCAM-1 in patients with normal urinary albumin/creatinine ratio after 3 months of treatment with liraglutide indicating progressive endothelial dysfunction. However there was no change in serum levels of sICAM-1 pre and post treatment. MCP-1 and hsCRP levels were unchanged after 3 months of treatment with liraglutide. There were no statistically significant changes in blood pressure, total cholesterol, LDL cholesterol or urinary albumin/creatinine ratios.

**Discussion**

In our cohort study the progression of diabetic kidney disease as evidenced by urinary albumin creatinine ratio was not influenced by liraglutide. Our regression model was robust as evidenced by $F$=2.10 which was statistically significant with p=0.036. After controlling for the effects of age, duration of diabetes, baseline blood pressures and baseline HbA1c, the most reliable predictor of change in albumin excretion in our cohort was the baseline urinary ACR. This is in agreement with previously published data on progression of ACR in subjects with normoalbuminuria (Forsblom, Groop et al. 1998, Poulsen, Ebbehøj et al. 1999, Yamada, Komatsu et al. 2005). Smoking and glycaemic control have also been noted to adversely influence progression of normoalbuminuria (Forsblom, Groop et al. 1998). In our retrospective cohort, the data on smoking status of subjects was incomplete; hence we were not able to confirm or refute this observation. In our study the influence of baseline HbA1c approached statistical significance with p=0.07.

Hypertension is closely linked with development of microalbuminuria in subjects with normal urinary ACR (1998, Adler, Stevens et al. 2003, Casas, Chua et al. 2005). In our study, the regression of urinary ACR was associated with reduction in diastolic blood pressure but no association with systolic blood pressure was evident. Previous studies in normoalbuminuric subjects have noted a correlation between urinary ACR and a blunted circadian variation in blood pressure (Poulsen, Ebbehøj et al. 1999). In one study, the nocturnal systolic blood pressure correlated with urinary ACR. But there was no correlation between ACR and daytime blood pressure or mean blood pressure(Lurbe, Redón et al. 1993). It is possible that subjects in our study who had progression of urinary ACR had significant nocturnal systolic hypertension. This could be a possible reason why the change in systolic blood pressure (recorded during clinic visit) was not a significant predictor of outcome in our cohort.

Our regression analysis of the data indicated that the total model was able to explain 19% of the change in urinary ACR at 12 months. Our model based on retrospective anthropometric and clinical biochemistry data was unable to explain the remaining 81% of variability. This suggests that a number of other factors that are not measured as part of routine clinical practice might be involved in influencing development of microalbuminuria. We hypothesised that biomarkers of endothelial dysfunction and low grade inflammation might be predictors of disease progression.

In a previously published prospective observational study, 192 patients with normoalbuminuria were followed up to a maximum of 9 years. It was noted that subjects with endothelial dysfunction (indicated by raised serum sICAM-1 and aVCAM-1 levels) were more likely to have progressive diabetic renal disease (Astrup, Tarnow et al. 2008). It has been shown that serum levels of MCP-1and hsCRP are closely associated with urinary albumin excretion in patients with normoalbuminuria and type 2 diabetes (Takebayashi, Matsumoto et al. 2006, Astrup, Tarnow et al. 2008). Animal studies have shown that MCP-1 plays a critical role in the inflammation of the kidney leading on to diabetic nephropathy (Chow, Nikolic-Paterson et al. 2006, Chow, Nikolic-Paterson et al. 2007). We hypothesised that liraglutide might modulate the above mentioned pathways, thereby influencing development of microalbuminuria in subjects with normal urinary albumin excretion.

The results as displayed above do not show any significant change in sICAM-1, MCP-1 or hsCRP levels after treatment with liraglutide. There was a small but significant change in serum sVCAM-1 levels. This needs to be interpreted with caution because of the lack of a control group in the prospective arm of our study. The reasons why the beneficial effect noted in rat models were not replicated in human studies are likely to be multifactorial.

In a rodent study, a radiolabelled compound specifically targeting the GLP-1 receptor was employed to image the GLP-1 receptor positive tissues in vivo. A very high bio distribution was noted in rat kidneys, pancreas and lung. In addition a large proportion of the ligand/receptor complex was retained in the kidney when compared with other tissues. The investigators noted that uptake in rat kidneys could not be specifically inhibited, indicating a mechanism that is not saturable (Gotthardt, Lalyko et al. 2006). The GLP-1 receptor in human kidneys is generally expressed at a low level (Körner, Stöckli et al. 2007). The relative abundance of the receptor might one reason why the renal effects noted in rat models were not replicated in our human study.

Even though human and rat GLP-1 receptors are similar, there is a 10% difference in the amino acid sequences of the protein between the two species (Baggio and Drucker 2007). As the GLP-1 analogues are designed to work on human GLP-1 receptor, they might interact differently with the rat GLP-1 receptor. There is also evidence that some of the effects of native GLP-1 in rats might be due to GLP-1 receptor independent mechanisms. A study on intact mouse blood vessels supports the existence of a vasodialatory signalling mechanism for GLP-1 that is independent of the known functional receptor. This vasodialatory response was thought to be mediated by a metabolite of GLP-1 acting via nitric oxide dependent cGMP release (Ban, Noyan-Ashraf et al. 2008). This receptor independent pathway might account for some of the effects on the renal vasculature of rats, thereby influencing diabetic nephropathy. Hence it is difficult to extrapolate the GLP-1 effects on rat models of nephropathy to human subjects with diabetes.

**Conclusion**

In conclusion, treatment with liraglutide does not have an influence on the development of microalbuminuria in subjects with type 2 diabetes and normal urinary albumin/creatinine ratios. In view of the possible protective effect of GLP-1 noted in rat models, it will be appropriate to repeat the experiments in higher mammals. As our knowledge of biomarkers that predict progression to microalbuminuria is incomplete, longitudinal human studies are required to evaluate reliable markers specific to diabetic kidney disease.

# CHAPTER 5

## EFFECT OF LIRAGLUTIDE ON DIABETIC RETINOPATHY IN PATIENTS WITH TYPE 2 DIABETES

## Introduction

Diabetic retinopathy is the most severe ocular complication of diabetes and usually manifests as gradual, painless progression of visual loss.(Coyne, Margolis et al. 2004, Frank 2004). Out of all the complications of diabetes, retinopathy has possibly the most substantial negative effect on activities of daily living(Fenwick, Pesudovs et al. 2011). Although advances in the management of diabetic retinopathy have reduced the risk of blindness, it still remains a significant problem because diabetes is a common disease.

Randomised controlled trials have shown that maintenance of near normal glycaemia and tight control of blood pressure contribute substantially towards slowing the progression of diabetic retinopathy (1993, Beulens, Patel et al. 2009). Laser retinal photocoagulation introduced in the 1960s substantially reduced the risk of severe visual impairment due to retinopathy. Pan-retinal photocoagulation used in the 'Diabetic Retinopathy Study' causes regression of new vessels in the retina without actually photocoagulating them(1981). Presumably, hypoxia in peripheral regions of the retina acts as a stimulus for neovascularisation, which is removed by photocoagulation. Focal laser treatment significantly reduced the progression of visual loss due to macular oedema in the 'Early Treatment Diabetic Retinopathy Study'(1991). Even though the mechanism of action of laser therapy is still hotly debated, it has become standard treatment for advanced diabetic retinopathy.

Increased expression of VEGF is currently thought to be the key mediator of diabetic retinopathy. VEGFs are a family of peptides produced from a single gene and are mitogenic for vascular endothelial cells and also increase permeability of the microvasculature(Frank 2004). It has been shown that hypoxia, which is a strong stimulus for neovascularisation, is associated with increased VEGF expression(Shweiki, Itin et al. 1992). In the presence of active neovascularisation, the levels of VEGF in vitreous humour are raised 20 times, when compared with normal eyes (Aiello, Avery et al. 1994). Anti-VEGF treatment is the most recent advance in the management of diabetic maculopathy and has shown promising results (Virgili, Parravano et al. 2012).

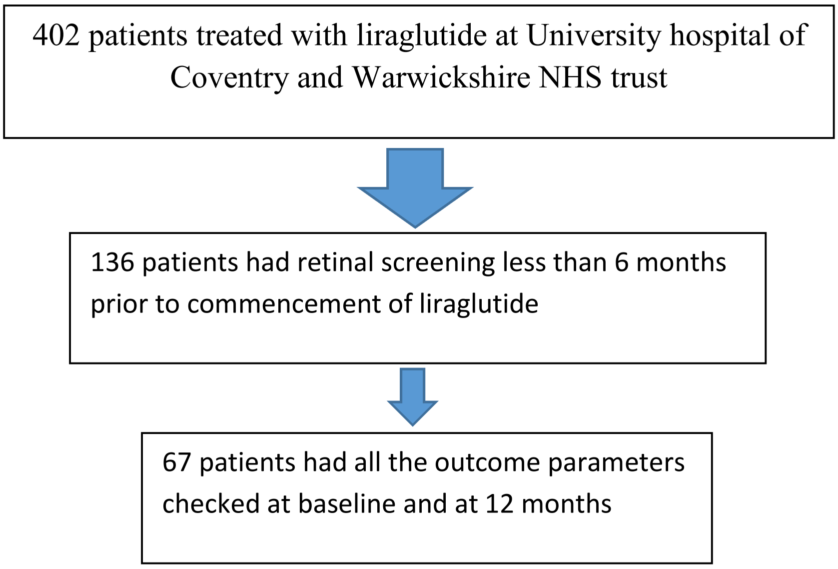
Animal studies suggest a possible protective role for GLP-1 in diabetic retinopathy (Zhang, Wang et al. 2009, Zhang, Zhang et al. 2011). However limited human studies suggest a possible adverse effect of GLP-1 based treatments on progression of retinopathy(Brooks and Lissett 2009).

**Aim**

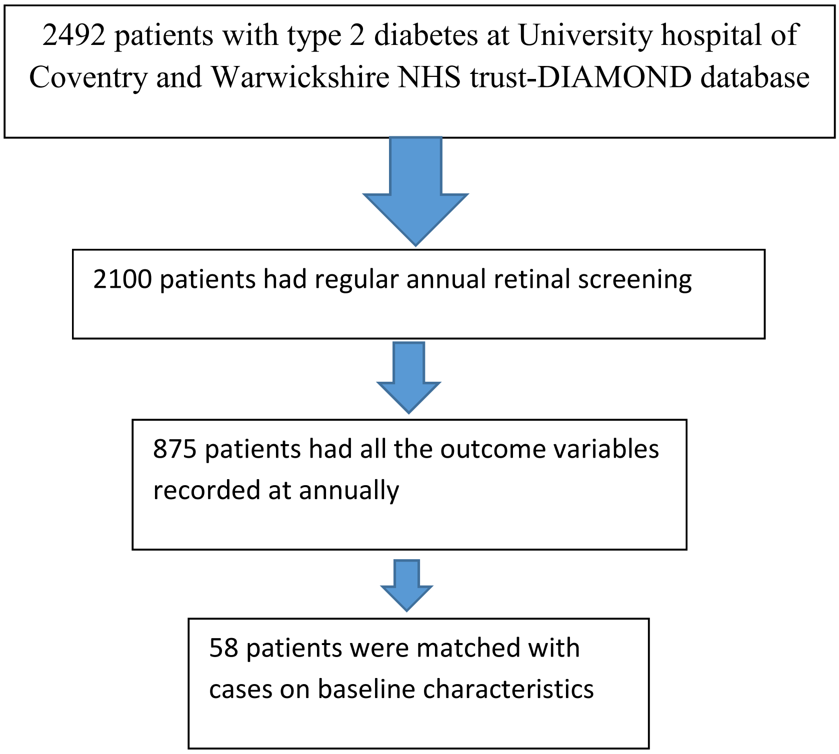
In this study, I have explored the effect of liraglutide on diabetic eye disease of subjects with type 2 diabetes.

**Methods**

From the database of patients at University hospital of Coventry and Warwickshire NHS trust, we identified the subjects treated with liraglutide, who had a retinal photograph in the 6 month period before starting the drug. From this group we identified the patients who continued liraglutide for at least 12 months and had a repeat retinal screening at the end of the 12 month period. The flowchart below depicts how cases were identified.




Matched controls were identified from the DIAMOND database at the hospital. The controls were patients with type 2 diabetes, not treated with liraglutide and had 2 retinal photographs at least 12 months apart. The flowchart below depicts selection of controls.




The retinopathy grades in both eyes of the cases and controls were collected from the OPTOMIZE database. The changes in retinopathy grades over 12 months were compared between cases and controls.

Primary outcome

1. Changes in retinopathy grades after 12 months when compared with baseline retinopathy grades.

Secondary outcome

1. Changes in BMI, HbA1c, total cholesterol, systolic and diastolic blood pressures of subjects after 12 months, when compared with baseline values.

All patients attending the GLP-1 clinic to commence treatment with liraglutide were invited to participate in the prospective study. Details of patient recruitment are detailed in chapter 2-Methods. Out of 42 patients, 19 patients agreed to participate. They were recruited in the prospective study after informed consent. Fasting plasma samples were collected from the subjects before and 3 months after treatment.

Each serum sample was assayed for the molecule of interest in duplicate and the mean of the two values was used for statistical analysis. If the duplicates varied by >10%, that particular sample was excluded from the analysis.

Primary outcome

**1.** Changes in plasma VEGF levels of subjects after 3 months of liraglutide treatment.

**Results**

In our retrospective cohort study, the retinopathy grades of 133 eyes (67 patients) were compared before, and 12 months after treatment with liraglutide. They were correlated with retinopathy grades of an appropriately matched control group of 116 eyes (58 patients) who had 2 retinal photographs at least 12 months apart. The baseline characteristics of the two groups of patients are shown on table 1.

| DOMAIN | LIRAGLUTIDE Mean (Standard deviation) | CONTROL Mean (Standard deviation) |
|---|---|---|
| **Number** | 133 | 116 |
| **Males:Females (number)** | 63:70 | 53:63 |
| **Duration of diabetes (years)** | 11 ± (7.87) | 11.81 ± (8.55) |
| **Age (years)** | 56.25 ± (9.24) | 58.53 ± (9.93) |
| **Body weight (kg)** | 111.52 ± (18.40) | 106.70 ± (19.94) |
| **Body mass index (kg/m2)** | 39.04 ± (5.47) | 37.95 ± (5.32) |
| **HbA1c (mmol/mol)** | 69.80 ± (16.24) | 68.00 ± (17.19) |
| **Systolic blood pressure (mm Hg)** | 147.81 ± (18.01) | 144.06 ± (16.14) |
| **Diastolic blood pressure (mm Hg)** | 80.06 ± (9.28) | 82.49 ± (10.85) |
| **Total cholesterol (mmol/l)** | 4.00 ± (0.84) | 4.39 ± (1.30) |
| **Urinary albumin/creatinine (mg/mmol)** | 4.59 ± (8.69) | 6.76 ± (11.90) |
| **Visual acuity (6/x)** | 7.23 ± (5.43) | 7.97 ± (6.81) |
| **R score (%)** | R0-57.9%<br>R1-41.4%<br>R2-0.8% | R0-54.3%<br>R1-40.5%<br>R2-5.2% |
| **M score (%)** | M0-93.2%<br>M1-6.8% | M0-93.1%<br>M1-6.9% |

Table 5.1: BASELINE CHARACTERISTICS OF SUBJECTS TREATED WITH LIRAGLUTIDE AND MATCHED CONTROLS

After 12 months change in the severity of retinopathy in each eye was scored as ‘improved’, ‘stable’ or ‘worse’. Retinopathy was defined as ‘improved’ if there was a reduction in the ‘R’ and/or ‘M’ grade. If there were no change in both ‘R’ and ‘M’ grades after 12 months, the eye was defined as ‘stable’. If there was an increase in the ‘R’ and /or ‘M’ grade, the eye was defined as ‘worse’. As laser therapy causes a significant change in retinopathy grades, eyes treated with photocoagulation during the 12 month period were excluded.

Normality of distribution of the baseline variables were assessed by Kolmogorov-Smirov and Shapiro-Wilk tests using SPSS software (threshold for significance was 0.05). If the ‘p’ values for both tests were non-significant, these variables were considered normally distributed for the purpose of this study.

Chi-square test for independence indicated no significant association between change in severity of retinopathy and treatment with liraglutide, $\chi^2$ (2, n= 249)=2.684, p=0.261, Cramer’s V=0.104. The results are shown on figure 1. At 12 months there were significant improvements in HbA1c and BMI of subjects treated with liraglutide as shown on figure 2. However, the changes in blood pressure and total serum cholesterol were not significant between the groups as shown on figure 3.

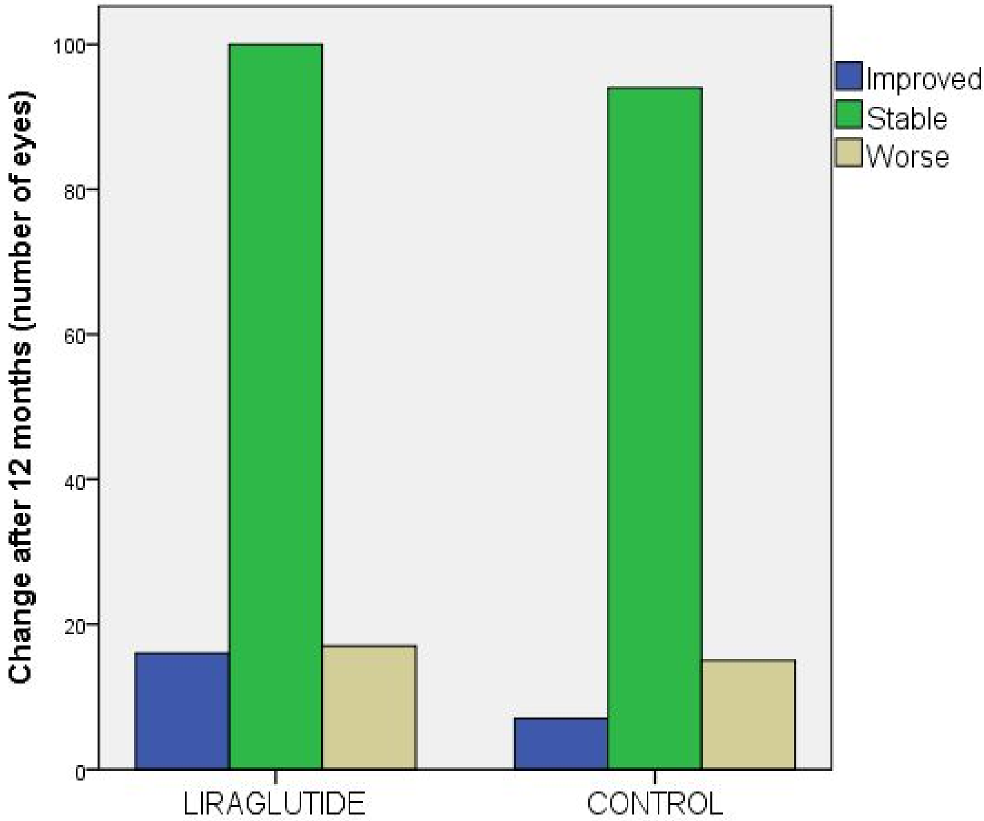



Figure 5.1: CHANGES IN SEVERITY OF RETINOPATHY AFTER 12 MONTHS. Chi-square test for independence did not detect any statistically significant association between change in severity of retinopathy and treatment with liraglutide.

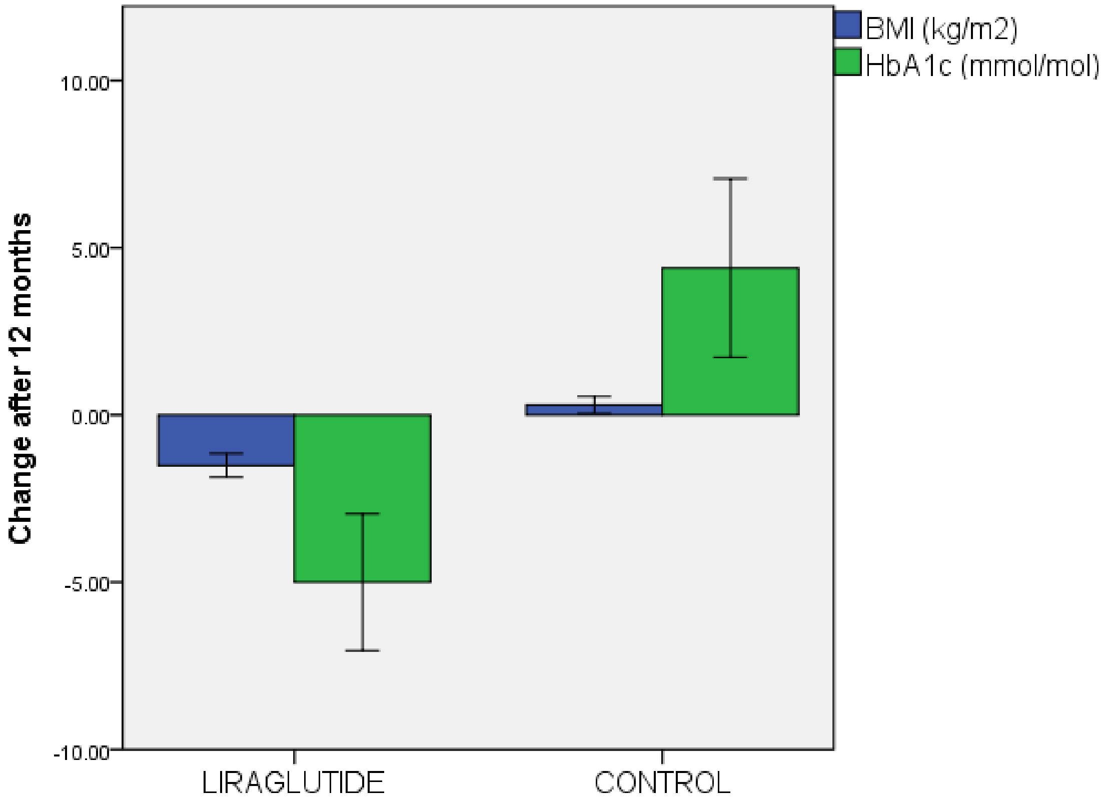



Figure 5.2: CHANGES IN BMI AND HBA1C OF CASES AND CONTROLS. Treatment with liraglutide for 12 months resulted in statistically significant improvements in BMI and HbA1c when compared with controls.

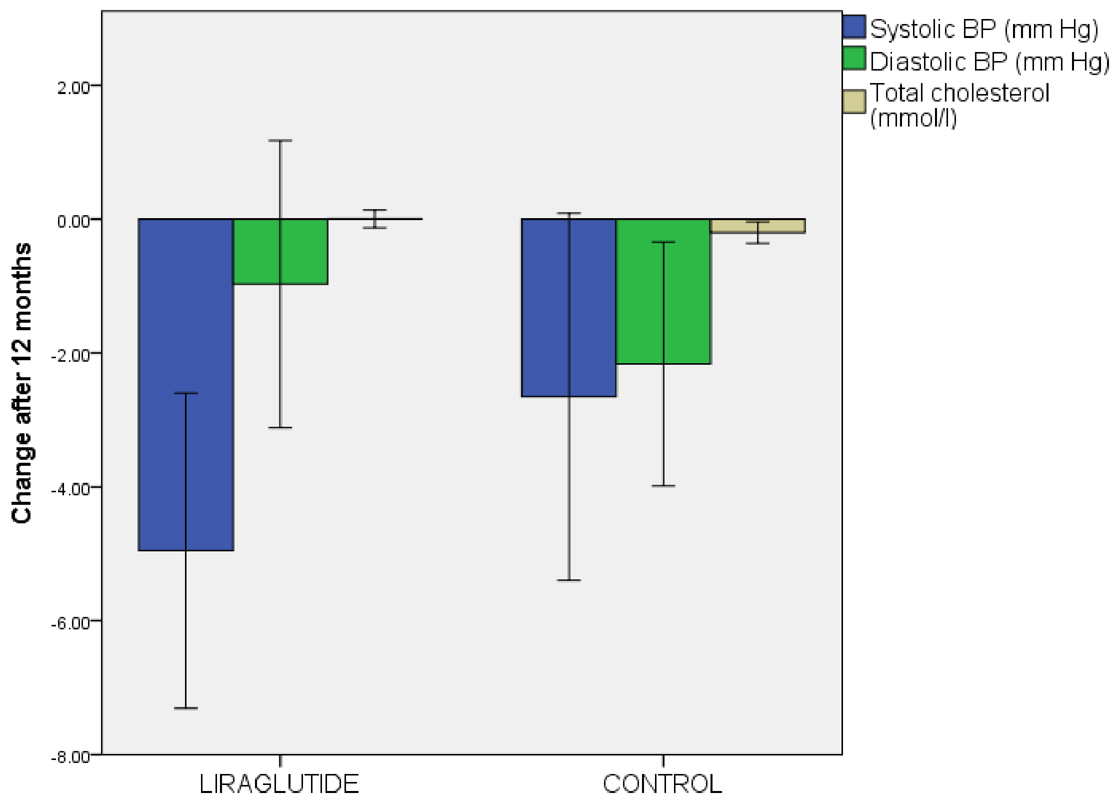



Figure 5.3: CHANGES IN BLOOD PRESSURE AND TOTAL SERUM CHOLESTEROL OF CASES AND CONTROLS. No statistically significant changes in total cholesterol levels or blood pressures were evident between the two groups.

We measured plasma VEGF levels of 19 patients with type 2 diabetes and varying grades of retinopathy before and 3 months after treatment with liraglutide. The baseline characteristics of the subjects are shown on table 2.

| DOMAIN | BASELINE CHARACTERISTICS<br>Mean (Standard deviation) |
|---|---|
| **Number of eyes** | 19 |
| **Males:Females** | 9:10 |
| **Number of eyes** | 38 |
| **Retinopathy grades** | R0=22<br>R1=10<br>R2=3<br>R3=3 |
| **Age**<br>**(years)** | 56.10 ± 10.45 |
| **Duration of diabetes**<br>**(years)** | 9.70 ± 6086 |
| **Urinary albumin/creatinine**<br>**(mg/mmol)** | 4.73 ± 6.63 |
| **Body Mass Index**<br>**(kg/m$^2$)** | 44.61 ± 14.00 |
| **HbA1c**<br>**(mmol/mol)** | 72.05 ± 18.07 |

Table 5.2: BASELINE CHARACTERISTICS OF SUBJECTS TREATED WITH LIRAGLUTIDE

When compared with baseline values, there were no significant changes in VEGF levels of the subjects after liraglutide treatment. The results are shown on figure 4. However significant improvements in HbA1c and BMI were noted.

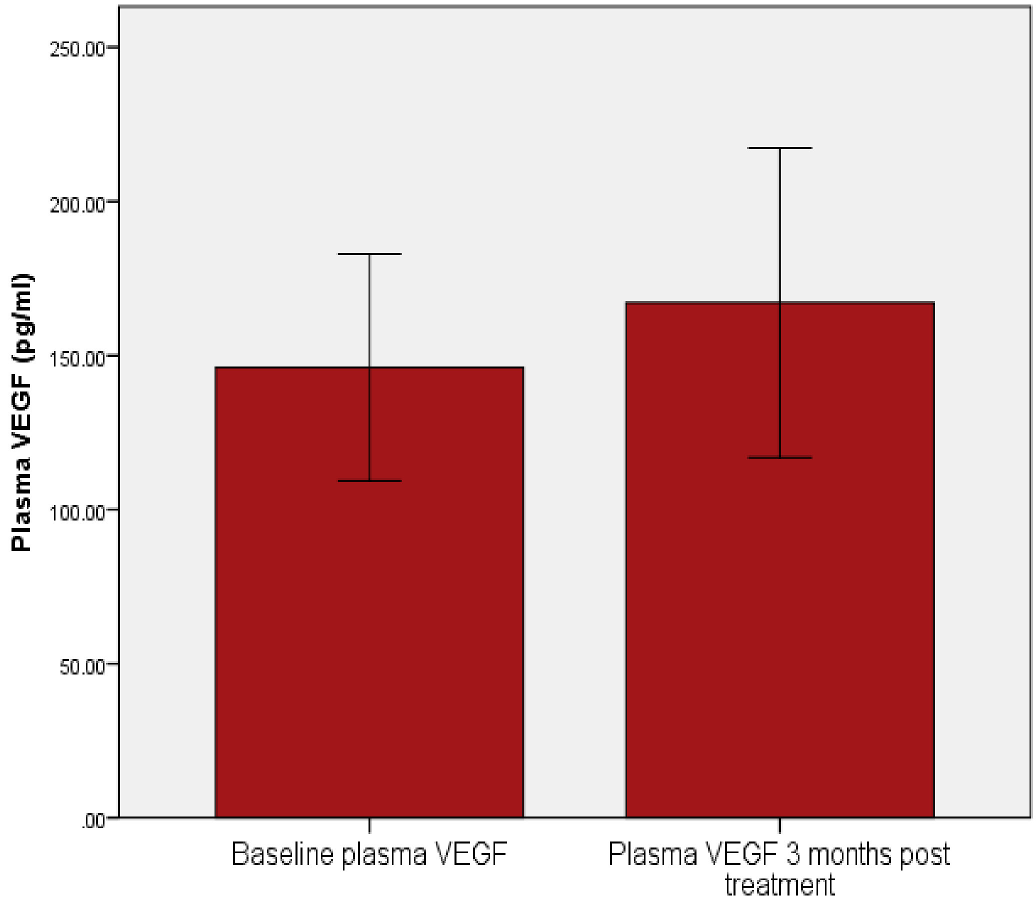



Figure 5.4: PLASMA VEGF LEVELS AT BASELINE AND 3 MONTHS POST TREATMENT IN SUBJECTS WITH TYPE 2 DIABETES. There were no statistically significant differences in mean circulating VEGF levels of the study cohort after 3 months treatment with liraglutide.

## Discussion

Our study has shown that treatment with liraglutide does not have a detrimental effect on diabetic retinopathy in subjects with type 2 diabetes. There is a case report of rapid improvement in glycaemic control caused by addition of GLP-1 analogue treatment resulting in worsening of diabetic retinopathy (Brooks and Lissett 2009). A retrospective analysis of patients treated with exenatide for at least 6 months was performed by Varadhan *et al* to assess its effect on retinopathy. Subjects with one retinal photograph before commencement of treatment and a repeat photograph taken at least 3 months after starting exenatide were included in this study. Worsening of retinopathy was noted in 29% patients, whereas an improvement was noted in 19% (Varadhan, Humphreys et al. 2011). To our knowledge this is the only published human study exploring the effect of GLP-1 analogues on retinopathy.

As acknowledged by the authors, the results had to be interpreted with caution because of lack of a control group. In addition the duration of follow ranged from 90 to 617 (mean 216) days. The worsening of retinopathy was thought to be due to rapid improvement in glycaemic control following exenatide treatment. In the Diabetes Complications and Control Trial (DCCT), it was noted that patients subjected to intensive insulin treatment had initial worsening of retinopathy in the first few months followed by an improvement (1998).

Hence, in our study we included a matched group of patients who were not treated with liraglutide as controls. We standardised the inclusion and exclusion criteria with regards to the timing of retinal photography as explained in detail in the methods section. We also specified that the second retinal photograph should be done at least 12 months after starting liraglutide to overcome the confounding effect of transient initial worsening of retinopathy. In our retrospective cohort study, wherein cases and controls were matched for baseline anthropometrics, clinical biochemistry and initial severity of retinopathy, treatment with liraglutide did not influence the course of diabetic retinopathy.

We performed sequential multivariate logistic regression to identify other parameters that might have an effect on retinopathy. The change in severity of retinopathy in each eye, scored as 'improved', 'stable' or 'worse' as mentioned above was defined as the dependent variable. Age, gender and duration of diabetes were entered as independent variables in step 1. In step 2 severity of retinopathy at baseline was entered as initial 'R', 'M' and 'P' grades in each eye. After step 2 the total model explained 16% of variability in the dependent variable. In step 3 baseline values and the changes in HbA1c, BMI, systolic and diastolic blood pressures were entered. After controlling for the above mentioned variables, treatment with liraglutide was entered in the final step.

The total model was robust, as evidenced by $F$ (16, 204)=3.035, $p<0.01$ and explained 19% of variability in progression of retinopathy. Whether the patient was treated with liraglutide or did not explain any additional variance, $F$ change (1, 204) = 0.729, $p=0.394$. In the final model, initial severity of retinopathy as measured by 'R' ($\beta=-0.33$, $p<0.01$), 'M' ($\beta=-0.25$, $p<0.01$) and 'P' ($\beta=0.208$, $p=0.04$) scores were statistically significant suggesting that initial severity of retinopathy has the biggest influence on its further progression or improvement.

It has been shown that baseline micro aneurysm counts can predict changes in severity of retinopathy over the next 4 to 10 years (Klein, Meuer et al. 1995). The epidemiology of diabetes complications study in which subjects were followed up for 2 years also noted that initial severity of retinopathy was a good predictor of progression (Lloyd, Klein et al. 1995). This is in agreement with our study where the median time interval between the two retinal photographs was 17.2 (range 13 to 21) months.

Previous studies have shown that better glycaemic control is associated with less severe retinopathy (Brinchmann-Hansen, Dahl-Jørgensen et al. 1992, 1998, Stratton, Kohner et al. 2001, Klein, Knudtson et al. 2008). The UKPDS is the longest intervention study to evaluate the effect of glycaemia on diabetic retinopathy and it clearly showed that improved blood glucose control reduced the risk of retinopathy by 25%. But the beneficial effects on retinopathy were evident only at the 10 year follow up(1998). In the ADVANCE retinal measurements study, 1602 patients with type 2 diabetes were followed up for 4.1 years. Intensive glycaemic control did not significantly reduce the incidence or progression of retinopathy, but a trend towards benefit was observed (Beulens, Patel et al. 2009). In our cohort, the change in HbA1c over a period of 12 months did not have statistically significant effect on progression of diabetic retinopathy; but there was a trend towards an association between lower baseline HbA1c and improvement in severity of retinopathy.

In some longitudinal studies, tight blood pressure control is also associated with better retinopathy outcomes, but the strength of association is less than with glycaemia (Matthews, Stratton et al. 2004, Klein, Knudtson et al. 2008). Again the association was more evident with longer duration of follow up. Among the 1148 subjects with hypertension in the UKPDS study, good control of blood pressure reduced the incidence and progression of retinopathy by 34%, but this effect was seen only after 7.5 years (Matthews, Stratton et al. 2004). In our regression analysis, blood pressure was not a significant predictor of change in severity of retinopathy. This observation is likely due to the relatively short duration of follow up in our study.

In our prospective study we measured serum VEGF as a biomarker for retinopathy which might indicate the course of retinopathy over time. Previous studies showed that serum and ocular VEGF levels are closely associated with severity of diabetic retinopathy and it has been proposed that serum cytokines such as VEGF can be used as a prognostic marker for retinopathy (Koleva-Georgieva, Sivkova et al. 2011, Mahdy and Nada 2011). Antiangiogenic therapy with anti-VEGF modalities is currently used as a treatment for diabetic retinopathy (Virgili, Parravano et al. 2012). In a prospective human trial, intravitreal injection of anti-VEGF drug bevacizumab caused a reduction in plasma VEGF levels before a significant change in retinopathy is seen (Ma, Zhang et al. 2012).

It is interesting to note that GLP-1 was found to be beneficial in animal models of retinopathy. GLP-1 receptor is expressed in the retina of rats predominantly in the inner layer. Exenatide administration has been shown to prevent retinal cell death and maintain normal retinal thickness in a rat model of diabetic retinopathy (Zhang, Wang et al. 2009). Intravitreal administration of exendin-4 in rats has been shown to protect the retina, functionally and morphologically from the insults of diabetes. The protective effect is thought to be due to up regulation of GLP-1 receptor and excitatory amino acid transporter in the Muller cells of the retina (Zhang, Zhang et al. 2011). GLP-1 produced by intravitreally implanted beads improved the survival of ganglion cells in rat retinae after an experimental optic nerve crush(Zhang, Zhang et al. 2011). To our knowledge, human studies have not been done to evaluate the role of the GLP-1 receptor in diabetic retinopathy.

I did not detect any significant change in serum VEGF levels of subjects with type 2 diabetes after treatment with liraglutide, suggesting that GLP-1 analogues do not influence retinopathy in humans. This is in agreement with the results of our retrospective cohort study.

**Conclusion**

Treatment with liraglutide does not have an adverse effect on progression of diabetic retinopathy in subjects with type 2 diabetes. This is in contrast to a previous uncontrolled study that suggested a possible detrimental effect of exenatide on diabetic retinopathy. In animal models, GLP-1 based treatments had a beneficial effect on retinopathy. However, in this first ever prospective human study there was no change in blood VEGF concentrations (biomarker for retinopathy) following treatment with liraglutide.

# CHAPTER 6

## EFFECT OF LIRAGLUTIDE ON BODY COMPOSITION AND MARKERS OF CARDIOVASCULAR RISK IN PATIENTS WITH TYPE 2 DIABETES AND MORBID OBESITY

## Introduction

The prevalence of obesity and type 2 diabetes are increasing globally as a result of high calorie diets and sedentary lifestyle that have become common in modern society (Fernández, Casazza et al. 2008). Unfortunately most therapies targeting hyperglycaemia cause weight gain which is associated with increased cardiovascular risk (1998, Purnell, Hokanson et al. 1998). Recently, excess adiposity and insulin resistance have been identified as novel predictors of cardiovascular risk independent of traditional risk factors such as BMI and blood pressure (Haffner, Mykkänen et al. 2000, Saely, Aczel et al. 2005). Therapies such as GLP-1 analogues that induce weight loss while improving glycaemic control have a beneficial influence on body composition of subjects with a mild degree of obesity.

Exenatide, the first GLP-1 analogue licenced to treat diabetes was noted to reduce body fat mass and improve markers of cardiovascular risk in subjects with mild obesity (Mean BMI 30 kg/m$^2$). Short term (20-28 days) treatment with liraglutide in human subjects with mild obesity showed conflicting effects on body composition (Harder, Nielsen et al. 2004, Horowitz, Flint et al. 2012). But in two randomised controlled trials lasting 26 to 52 weeks, liraglutide induced significant reductions in total body fat and percentage of body fat(Garber, Henry et al. 2009, Jendle, Nauck et al. 2009, Nauck, Frid et al. 2009). The average BMI of subjects in the two studies were 30 kg/m$^2$ and 33 kg/m$^2$ respectively. The effect of liraglutide on body composition and cardiovascular risk markers has not been studied in subjects with morbid obesity defined as BMI > 40 kg/m$^2$.

## Aim

The aim of our study was to evaluate whether subjects with type 2 diabetes and morbid obesity treated with liraglutide had a change in total and regional adiposity. We also assessed the effect on glucose-insulin homeostasis in these subjects as a marker of cardiovascular risk in addition to other clinical parameters.

## Methods

All subjects attending the GLP-1 clinic at University hospital of Coventry and Warwickshire were invited to participate in this prospective study. The details of the study were explained to the patients by the principal investigator or a member of the research team. Patients were requested to consider participation after reflection for at least 24 hours. Out of 43 patients, 20 subjects (10 men and 10 women) with morbid obesity and type 2 diabetes agreed to participate.

Body composition analyses at baseline and 3 months after treatment were performed using InBody 720 analyser as described in the methods section. Blood tests and anthropometric data were collected at baseline and at follow up. HOMA2 calculator software (Diabetes trials unit, University of Oxford) was employed to calculate insulin sensitivity and beta cell function.

Each serum sample was assayed for the molecule of interest in duplicate and the mean of the two values was used for statistical analysis. If the duplicates varied by >10%, that particular sample was excluded from the analysis.

Primary outcomes

1. Changes in BMI, fat mass an lean body mass after 3 months of treatment with liraglutide
2. Changes in insulin sensitivity measured as HOMA%S

**Results**

The baseline and 3 month follow up data on anthropometrics and clinical markers of cardiovascular risk are shown on table 1. The average age of patients in our study was 56.1 years (standard deviation 10.4). All subjects completed the three month study. Data was analysed using SPSS software version 21. Paired samples‘t’ test was employed to compare mean changes in continuous variables at baseline and 3 months after treatment.

| DOMAIN<br>n=20 | BASELINE<br>Mean (Standard Deviation) | 3 MONTHS AFTER LIRAGLUTIDE<br>Mean (Standard Deviation) | CHANGE AFTER 3 MONTHS<br>Mean (95% Confidence intervals) | Significance. p= |
|---|---|---|---|---|
| **Body weight (kg)** | 127.19 (41.80) | 123.14 (38.07) | -4.04 (-1.52 to -6.56) | 0.01 |
| **Waist circumference (cm)** | 125.90 (19.90) | 121.14 (16.98) | -4.76 (-1.99 to -7.52) | 0.01 |
| **Waist:Hip ratio (ratio)** | F 0.94 (0.06)<br>M 1.05 (0.08) | F 0.92 (0.07)<br>M 1.03 (0.09) | F -0.02 (-0.01 to -0.03) M -0.02(-0.02 to +0.01) | 0.008<br>0.155 |
| **Systolic blood pressure (mm Hg)** | 149.74 (19.38) | 139.63 (17.56) | -10.10 (-1.37 to -18.83) | 0.03 |
| **Diastolic blood pressure (mm Hg)** | 77.26 (10.61) | 72.53 (13.29) | -4.73 (+0.47 to -9.94) | 0.07 |
| **Average insulin dose over 24 hours (n=10) (units)** | 68.52 (96.21) | 40.19 (60.86) | -28.33 (-2.58 to -54.07) | 0.03 |
| **HbA1c (mmol/mol)** | 72.05 (18.77) | 58.19 (11.74) | -13.85 (-7.30 to -20.40) | 0.01 |
| **Total cholesterol (mmol/l)** | 4.21 (1.02) | 3.92 (1.09) | -0.28 (+0.07 to -0.64) | 0.11 |
| **HDL cholesterol (mmol/l)** | 1.18 (0.37) | 1.14 (0.26) | -0.03 (+0.05 to -0.12) | 0.44 |
| **LDL cholesterol (mmol/l)** | 2.08 (0.65) | 1.99 (0.87) | -0.09 (+0.20 to -0.39) | 0.51 |
| **Triglycerides (mmol/l)** | 2.07 (1.15) | 1.74 (0.85) | -0.32 (+0.04 to -0.61) | 0.03 |

Table 6.1: CLINICAL AND BIOCHEMICAL PARAMETERS AT BASELINE AND 3 MONTHS AFTER TREATMENT WITH LIRAGLUTIDE IN 20 HUMAN SUBJECTS

A significant reduction in average BMI from 44.97 kg/m$^2$ to 43.68 kg/m$^2$ (mean difference 1.28 kg/m$^2$; 95 % confidence intervals 0.26 to 2.26, p=0.013) was seen after 3 months. A reduction in percentage of body fat from 46.60% to 45.83% (mean difference 0.77%; 95 % confidence intervals 0.25 to 1.29, p=0.006) was also noted in these patients. These results are illustrated on chart 1.

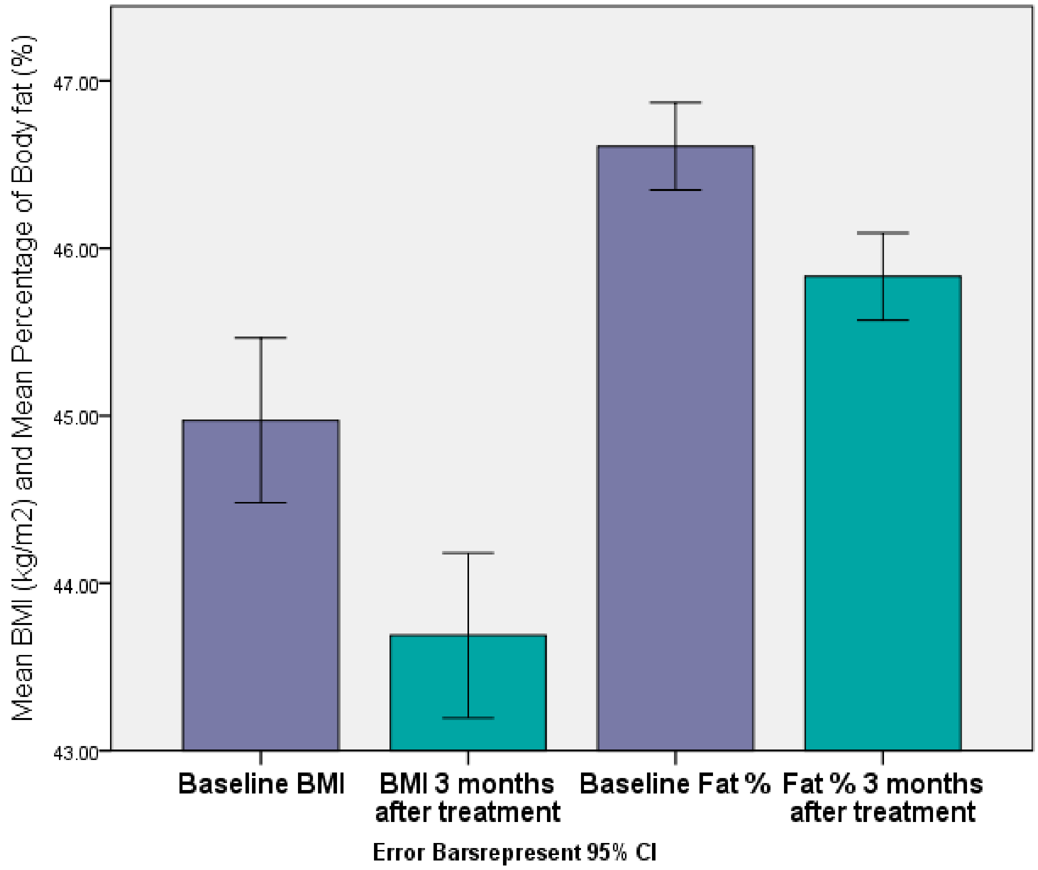



Figure 6.1: CHANGES IN BMI AND PERCENTAGE OF BODY FAT IN SUBJECTS TREATED WITH LIRAGLUTIDE FOR 3 MONTHS. Statistically significant improvements were noted in BMI and estimated percentage of body fat after 3 months of treatment with liraglutide.

We also noted a significant reduction in the mean total fat mass of these patients from 60.55 kg at baseline to 58.01 kg after 3 months (mean difference 2.54 kg; 95 % confidence intervals 1.27 to 3.81, $p=0.001$). But the change in mean lean body mass from 63.22 kg to 62.07 kg during the same period was not statistically significant (mean difference 1.14 $kg/m^2$; 95 % confidence intervals -0.63 to 2.92, $p=0.192$). The results are shown on chart 2.

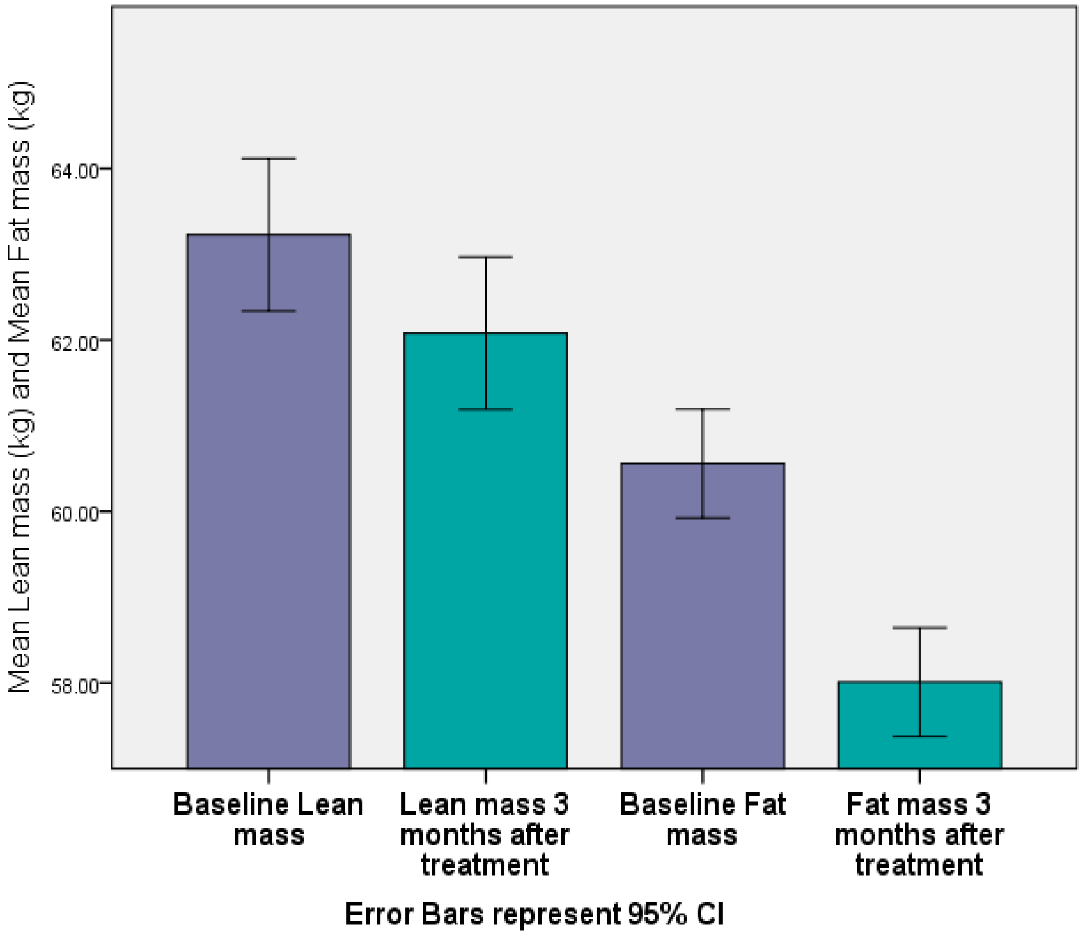



Figure 6.2: CHANGES IN MEAN LEAN BODY MASS AND FAT MASS IN SUBJECTS TREATED WITH LIRAGLUTIDE FOR 3 MONTHS. The changes in mean lean body masses were not statistically significant after 3 months of treatment with liraglutide. However significant improvements were noted in fat mass after similar duration of treatment.

We calculated the insulin sensitivity of the subjects using HOMA2 calculator software obtained from the Diabetes trials unit at University of Oxford. The improvement in mean insulin sensitivity expressed as a percentage (the output of the model is calibrated to give normal insulin sensitivity as 100% in a population) correlated with reduction in percentage of body fat even after controlling for change in BMI($r$=0.597, $p$=0.024). The results are shown on chart 3.

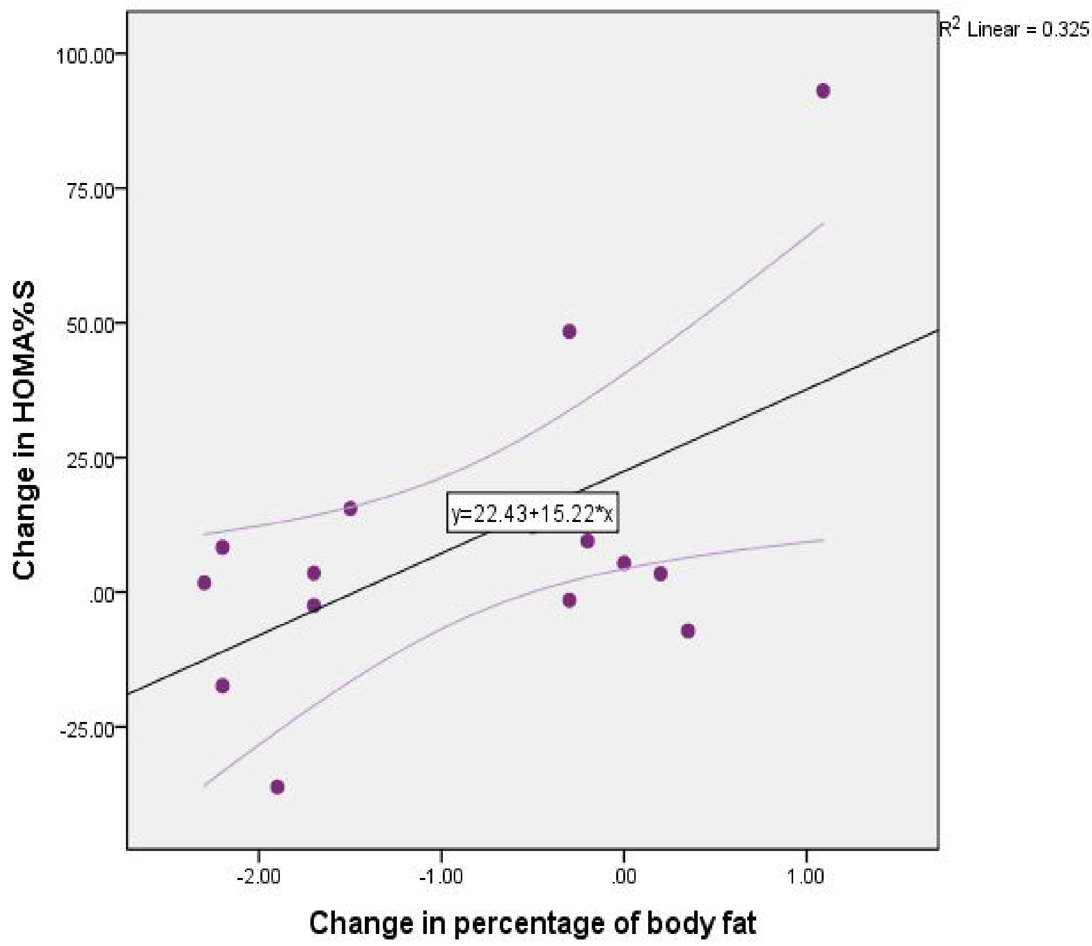



Figure 6.3: RELATION BETWEEN CHANGES IN HOMA-INSULIN SENSITIVITY AND PERCENTAGE OF BODY FAT. A statistically significant correlation was noted between changes in insulin sensitivity and changes in percentage of body fat. Changes in insulin sensitivity did not correlate with changes in BMI or changes in lean body mass.

## Discussion

This is the first study exploring the influence of liraglutide on body composition and glucose-insulin homeostasis in subjects with morbid obesity. Our results show that liraglutide treatment is associated with a significant reduction in BMI, which is mainly due to reduction in fat mass. In our subjects there was preservation of lean body mass even after significant weight loss. Hence, we did not observe any significant change in estimated basal metabolic rate (BMR) of our patients. It has been shown that, even in normal weight subjects excess adiposity is linked with a raised LDL cholesterol, raised triglycerides and low HDL cholesterol(Ito, Nakasuga et al. 2003). In a large cross sectional study involving 3800 subjects, percentage of body fat, rather than BMI was associated with cardiovascular risk. This association was seen after adjusting for age, gender, lifestyle and family history of obesity(Zeng, Dong et al. 2012). In our cohort, the improvement in percentage of body fat was associated with changes in traditional markers of cardiovascular risk, such as blood pressure.

In addition to changes in total body fat, we observed changes in regional fat distribution. There was a significant reduction in waist:hip ratios (WHR) of women after weight loss induced by liraglutide, but the change was not statistically significant in men. A number of large studies have identified WHR as an important predictor of cardiovascular risk (Dalton, Cameron et al. 2003, Welborn, Dhaliwal et al. 2003, Price, Uauy et al. 2006). In obese women higher cardiovascular risk was associated with central or 'android' obesity (WHR >0.86) than 'gynoid' obesity (WHR<0.86). Following liraglutide therapy, we observed a change from ‘android’ to ‘gynoid’ obesity in our cohort, which is associated with cardiovascular benefit(Crepaldi, Belfiore et al. 1991).

We also noted an absolute reduction in abdominal fat mass as evidenced by a statistically significant reduction in waist circumference (result shown on table 1). But the visceral fat area (VFA) measured by bio-impedance remained unchanged after treatment with liraglutide for 3 months. The mean VFA of the subjects was 213.52 $cm^2$ at baseline and 212.60 $cm^2$ after 3 months (change in VFA -0.91, 95% confidence interval 7.06 to -8.90, $p$=0.81). This suggests that change in abdominal fat in our cohort was mainly due to reduction in subcutaneous abdominal fat, rather than visceral fat.

In a previous study, liraglutide treatment of subjects with mild degree of obesity (BMI 30-33 $kg/m^2$) induced a significant improvement in both visceral and subcutaneous adipose tissues were noted (Jendle, Nauck et al. 2009). It is possible that visceral adipose tissue in patients with morbid obesity is less susceptible to weight loss interventions. The expression of genes related to cell cycling, membrane transport and energy metabolism is different in the adipose tissues of morbidly obese subjects, when compared with controls(Baranova, Collantes et al. 2005). The reason why our subjects did not lose any significant visceral adipose tissue could be due to inherent differences in cell turnover as a result of morbid obesity.

Instruments such as InBody 720 that employ the technique of Bio impedance Spectroscopy are able to provide estimation of fat mass, lean body mass, skeletal mass and water content of inta-cellular and extra-cellular compartments (Heymsfield, Wang et al. 1996, Nuñez, Gallagher et al. 1997). This technique is preferred by investigators in view of portability, ease of use, relatively low cost, minimal participant participation and safety (Rush, Chandu et al. 2006).

Head to head comparison studies have noted that Bio impedance techniques provided values that compared favourably with values obtained by dilution technique for fat mass and fat free mass (Papathakis, Rollins et al. 2005). Bio impedance values for fat free mass were underestimated in normal weight individuals and overestimated in overweight individuals when compared with DEXA (Ward, Dyer et al. 2007). CT scanning, Air displacement plethysmography and dilution techniques are less frequently used techniques for estimation of body composition (Clasey and Gater 2005, Noreen and Lemon 2006, MacNeil and Boyd 2007, MacNeil and Boyd 2007).

It is important to recognize that no single method of estimating body composition can be considered as gold-standard. Repeated measurements using the same technique over time can give valuable information over temporal changes in a cohort (Lee and Gallagher 2008).

Changes in glucose-insulin homeostasis as assessed by HOMA were evident in our patients and were mainly due to changes in total body fat. Matthews *et al* in 1985, first described homeostatic model assessment (HOMA) of insulin resistance and β cell function (Matthews, Hosker et al. 1985). In the basal state, the relationship between blood glucose and insulin reflects the balance between hepatic glucose output and insulin secretion from the pancreatic β cells(Turner, Holman et al. 1979). The predictions used in HOMA are based on data from experimental studies on humans. Its most recent version HOMA2, has non-linear solutions and takes into account variations in hepatic and peripheral glucose resistance (Rudenski, Matthews et al. 1991, Levy, Matthews et al. 1998). The computer model can be used to determine insulin sensitivity as %S (normal sensitivity is 100%) and β cell function as %B (normal β cell function is 100%) from paired fasting glucose and insulin measurements. This model allows for insulin sensitivity to be assessed in individuals treated with insulin as long as the blood sampling is done when glucose and insulin concentrations are in a steady state(Wallace, Levy et al. 2004). However the HOMA model is not suitable for assessment of β cell function in patients treated with exogenous insulin.

The safety and efficacy of liraglutide were assessed in the Liraglutide Effect and Action in Diabetes (LEAD) programme that comprised of six randomised controlled trials. These trials compared liraglutide with a range of anti-diabetic drugs as monotherapy and combination therapy. Data from these trials have shown conflicting effects of liraglutide on insulin resistance, in spite of consistent improvements in BMI. A proportion of participants in LEAD-2 and LEAD-3 trials had body composition analyses that showed significant reduction in fat mass with liraglutide (Jendle, Nauck et al. 2009). In LEAD-3 trial, a significant improvement in insulin sensitivity was observed in the liraglutide treated subjects, but this was not seen in the LEAD-1 and LEAD-2 trials (Garber, Henry et al. 2009, Marre, Shaw et al. 2009, Nauck, Frid et al. 2009). In LEAD-4 trial a significant improvement in insulin sensitivity was noted in both the liraglutide treatment group and the placebo group, but no difference was seen between groups (Zinman, Gerich et al. 2009).

In our study the mean insulin sensitivity improved from 37.08% at baseline to 44.73%, 3 months after liraglutide treatment, but the change was not statistically significant. The improvement in insulin sensitivity in our cohort correlated with changes in BMI and in percentage of body fat. The correlation with the latter was more pronounced and persisted even after statistically controlling for changes in BMI. It is possible that a bigger cohort of patients and/or longer follow we might observe a clear trend in insulin sensitivity.

**Conclusion**

Patients with type 2 diabetes are at risk of cardiovascular events, and the risk is magnified by coexistent morbid obesity. In these subjects, treatment with liraglutide for 3 months is associated with significant improvements in surrogate markers of cardiovascular risk such as percentage of body fat and waist:hip ratio. Human studies with hard cardiovascular end points are required to further explore the effects of GLP-1 based treatments on macrovascular disease associated with diabetes.

# CHAPTER 7

# GENERAL DISCUSSION AND CONCLUSION

# Summary of background

## Diabetes mellitus

Diabetes mellitus is an important cause of mortality and morbidity globally due to its direct clinical sequelae and chronic complications (1998, 2000). Type 2 diabetes is the most common form of diabetes worldwide. It is associated with mortality due to premature cardiovascular disease and morbidity due to blindness, amputation, and renal failure (Colberg, Sigal et al. 2010). It is clear that the prevalence of type 2 diabetes is rising globally fuelled by population growth and ageing in addition to the increasing incidence of obesity (Danaei, Finucane et al. 2011).

## Chronic complications of diabetes

The earliest functional change in the kidney due to diabetes is the appearance of glomerular hyper-filtration, which is followed by albuminuria. Patients with micro albuminuria are referred to as having incipient nephropathy (Bakker 1999). In a proportion of patients structural changes in the kidney follow leading on to progressive kidney injury (Molitch, DeFronzo et al. 2004). Hence, it is recommended that patients with type 2 diabetes are screened for microalbuminuria at diagnosis. Good glycaemic control and management of hypertension are well established treatment strategies to slow the progression of albuminuria (1998, 1998). Recent evidence suggests that low grade inflammation and endothelial dysfunction are responsible for initiation and progression of micro albuminuria (Furuta, Saito et al. 1993, Chow, Nikolic-Paterson et al. 2007). Indeed, studies on animal models suggest that blocking certain inflammatory pathways can improve urinary albumin excretion (Chow, Nikolic-Paterson et al. 2005). Ongoing research is focussed on drugs that can modulate these inflammatory pathways, and thereby improve albuminuria.

The estimated crude prevalence rate of diabetic retinopathy in a population of patients with any type of diabetes is about 40%, out of which 8% are likely to have sight threatening retinopathy (Kempen, O'Colmain et al. 2004). Management of diabetic retinopathy is based on achieving good glycaemic control and treatment of hypertension (1993, 1998). Laser photocoagulation is an established therapy for advanced diabetic retinal disease (1981, 1991). It has been recognised that inflammatory pathways, mediated by VEGF are implicated in the pathogenesis of retinopathy (Aiello, Avery et al. 1994).

It is well recognised that patients with type 2 diabetes are at risk of having adverse cardiovascular events (Mokdad, Bowman et al. 2001, Cleary, Orchard et al. 2006, Forbes and Cooper 2013). Management of type 2 diabetes not only involves addressing hyperglycaemia, but also encompasses appropriate therapy targeted at optimising other cardiovascular risk factors such as, hypertension, hypercholesterolemia and hypertriglyceridemia (2012). Recently it has been recognised that body composition, insulin resistance and body fat distribution are equally significant markers of cardiovascular risk (Kissebah, Vydelingum et al. 1982, Segal, Dunaif et al. 1987, Haffner, D'Agostino et al. 1999, Rewers, Zaccaro et al. 2004). Hence an ideal drug to treat diabetes should also have a beneficial effect on these cardiovascular risk factors, in addition to achieving glycaemic control.

**Glucagon like peptide-1**

GLP-1 is a gut derived insulinotropic hormone, which is secreted in response to hyperglycaemia and food intake (Holst and Orskov 2001). The receptor for GLP-1 is widely distributed in the human body, hence the actions of this hormone are not confined to the pancreatic β cell (Körner, Stöckli et al. 2007). Evidence form animal studies indicate that GLP-1 is anti-inflammatory and protects against endothelial dysfunction (Arakawa, Mita et al. 2010, Koska, Schwartz et al. 2010, Goto, Nomiyama et al. 2011). In animal models, GLP-1 and its analogues had a beneficial effect on diabetic nephropathy and diabetic retinopathy (Moreno, Mistry et al. 2002, Zhang, Wang et al. 2009, Zhang, Zhang et al. 2011, Hendarto, Inoguchi et al. 2012). To date, human studies have not evaluated the effect of GLP-1 analogues on microvascular complications of diabetes.

## Liraglutide and microalbumnuria

In this retrospective cohort study, I compared a group of patients with type 2 diabetes who were treated with liraglutide for up to 12 months, with a matched group of controls not treated with the drug. The changes in urinary albumin excretion rates and changes in retinopathy grades between the groups were compared, to detect whether liraglutide influences the course of microvascular complications. This is the first human study to explore the effect of liraglutide on microvascular complications of diabetes.

A prospective randomised controlled trial would be the ideal method to investigate the effect of a drug. However data from well-designed retrospective studies can give valuable insight and provide preliminary data to design a clinical study. A well-recognised weakness inherent to retrospective analyses is that the data available is often incomplete. In this study, I had to exclude about 40% of patients from the analysis because of incomplete data.

It is possible that improvement/worsening of microvascular complications are reflected in serum biomarkers before a change in clinical parameters is evident. Hence, I designed a prospective arm of the study to measure biomarkers of nephropathy, retinopathy and cardiovascular risk in subjects with type 2 diabetes before starting liraglutide. These biomarkers and clinical parameters were measured under standard conditions 3 months after treatment with liraglutide. A limitation of this prospective study was the lack of a matched control group of patients who were not treated with GLP-1 analogues.

In patients with type 2 diabetes and established microalbumnuria, treatment with liraglutide for 6-12 months did not have a significant impact on urinary albumin excretion rate. However, patients treated with liraglutide had a significant improvement in their HbA1c and BMI. Regression analysis revealed that baseline urinary albumin/creatinine ratio, HbA1c, and blood pressure were predictive of changes in urinary albumin excretion, which is in keeping with previously published studies. As the rate of progression of microalbumnuria is gradual, studies with a longer follow up duration might be required to determine conclusively, whether liraglutide has an influence on urinary albumin excretion. We intend to continue this study by following up this cohort of patients prospectively.

I conducted a similar retrospective analysis in patients with detectable urinary albumin excretion, but not diagnostic of pathologic microalbumnuria (as defined by the NICE criteria). The changes in urinary albumin excretion rated were not different between the cohort of patients treated with liraglutide and matched control subjects. As the initial urinary albumin/creatinine ratio was low, it is possible that the subsequent change after 12 months was not large enough to be detectable.

In a prospective study, I compared serum concentrations of the biomarkers of inflammation (MCP-1 and hsCRP) and endothelial dysfunction (sICAM-1 and sVCAM-1) that are closely associated with development and progression of diabetic nephropathy in subjects before and 3 months after treatment with liraglutide. Liraglutide therapy did not induce any change in these biomarkers. Although, I did not show any change in biomarkers in this human study, a number of studies in rodents have shown significant improvements in these biomarkers and urinary albumin excretion following administration of liraglutide (Moreno, Mistry et al. 2002, Kodera, Shikata et al. 2011, Hendarto, Inoguchi et al. 2012).

The expression of GLP-1 receptor in human kidneys is lower than the expression in renal tissues of rodents, which could explain the above noted results (Körner, Stöckli et al. 2007). Another possible reason could be the differing affinities of liraglutide to the GLP-1 receptor in humans and rodents due to the 10% differences in the amino acid sequences between the two species (Baggio and Drucker 2007).

## Liraglutide and diabetic retinopathy

In this retrospective study, treatment with liraglutide for 12 months did not induce any significant changes in the retinopathy grades of patients after controlling for other known factors that influence the course of diabetic retinopathy. A previous retrospective audit raised concerns about risk of progression of retinopathy following treatment with the GLP-1 analogue, exenatide (Varadhan, Humphreys et al. 2011). However these results have to be interpreted with caution, as the study did not include a group of patients not treated with exenatide to act as control subjects.

In my study, the group of patients treated with liraglutide had a significant reduction their mean HbA1c levels, but no improvements in the retinopathy grades were evident. However a trend towards an association between lower baseline HbA1c and improvement in retinopathy grades was noted. As noted in the UKPDS study and confirmed by other studies, the improvement in retinopathy lags behind the improvement in HbA1c by at least 4 years (1998, Matthews, Stratton et al. 2004, Beulens, Patel et al. 2009). It is possible that if the cohorts of patients in my study are followed up prospectively, more information might be available about the effects of glycaemic control and GLP-1 analogue treatment on retinopathy.

Studies conducted on rodents showed an improvement in diabetic retinopathy following treatment with GLP-1 analogues (Zhang, Wang et al. 2009, Zhang, Zhang et al. 2011). We conducted the first prospective human study involving 20 subjects with type 2 diabetes and varying degrees of retinopathy grades to evaluate the effect of liraglutide on retinopathy. There were no significant changes in the circulating concentrations of VEGF, which is a surrogate marker for the severity of diabetic retinopathy.

## Influence of liraglutide on body composition

As the effects of liraglutide on body composition and insulin sensitivity have not been explored in subjects with morbid obesity (who are group of patients with the highest cardiovascular risk), I conducted a prospective study involving 20 patients with type 2 diabetes and morbid obesity. After 3 months of treatment with liraglutide, significant reductions in total body fat mass and abdominal fat were noted, without any significant change in lean body mass. The decline in abdominal fat was mainly due to changes in abdominal subcutaneous fat, with little change in visceral fat (as estimated by bio-impedance). In a study involving subjects with a mild degree of obesity, significant improvements in both subcutaneous and visceral fat were noted after treatment with liraglutide (Jendle, Nauck et al. 2009). It is possible that visceral adipose tissue in subjects with morbid obesity is more 'resistant' to the weight reducing effects of liraglutide. It will be interesting to see the changes in visceral fat areas of subjects treated with liraglutide for 6 and 12 months.

The mean insulin sensitivity of this cohort of patients showed an improvement following liraglutide treatment; however this failed to reach statistical significance. In spite of consistent improvements in BMI following treatment with liraglutide, the effects on insulin sensitivity have been inconsistent in various trials (Garber, Henry et al. 2009, Jendle, Nauck et al. 2009, Marre, Shaw et al. 2009, Zinman, Gerich et al. 2009).

## Confounding factors and limitations of the studies

1. In the retrospective cohort study, matching of the cohorts was done at baseline. The group of patients treated with GLP-1 analogues had statistically significant improvements in HbA1c and BMI, when compared to the controls. This might have influenced the course of microvascular complications and cardiovascular risk.
2. About 40% of potentially eligible subjects were excluded from the study during the screening process due to incomplete datasets. This might have influenced the outcome. This drawback is inherent to all retrospective studies.
3. There was no control group for the prospective biomarker study.
4. All subjects included (cases and controls) had clinically significant obesity with BMI >35 kg/m$^2$. Therefore the results may not be generalizable to subjects without obesity

## Future research

It remains possible that there might be subtle effects of liraglutide on microvascular complications, which were not evident in this study. To answer this question conclusively, longer term prospective studies are needed.

It is planned that our cohort of subjects on liraglutide will be followed up prospectively, as well as the controls. Data on all the above mentioned parameters will be collected at 6 monthly intervals and similar analyses done at 6 monthly intervals. If significant differences are noted in the rates of progression of urinary albumin/creatinine ratio and/or retinopathy are noted, the next step will be to design a prospective randomised multicentre study to further clarify the role of GLP-1 analogues in diabetes complications.

Currently it is not possible to do power calculations to design a prospective study because we were not able to identify any significant differences between out treatment and control groups. However, we expect further data which might be available on continued follow up of our cohort to guide us in making power calculations.

## Conclusion

In patients with type 2 diabetes and morbid obesity, treatment with the GLP-1 analogue, liraglutide induces a significant reduction in total body fat, without changing lean body mass. This was associated with improvements in markers of cardiovascular risk and a trend towards enhanced insulin sensitivity.

In study on human subjects with type 2 diabetes, treatment with the GLP-1 analogue liraglutide did not influence the onset/progression of microalbuminuria. The course of diabetic retinopathy in patients with type 2 diabetes was not influenced by treatment with liraglutide.

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